# MALARIA POLYPEPTIDES

## Technical field

The present invention relates to carbohydrates capable of acting as receptors for
malaria antigens present on the surfaces of malaria infected erythrocytes. In addition,
the invention also relates to novel malaria polypeptides capable of acting as ligands in
relation to the receptors according to the invention. The invention also encompasses
the use thereof as medicaments and vaccines, pharmaceutical compositions containing
the same as well as antibodies directed against said new ligands.

## Background and prior art

On a worldwide basis, malaria is one of the most common infectious diseases. Even
though it has been largely eliminated from North America and Europe, it remains the
most serious infectious disease in tropical and subtropical regions of the world.
According to the World Health Organisation, WHO, there are about 100 million new
cases each year, and about 300 million people in the developing countries exhibits
chronical malaria infections. Malaria is caused by four species of *Plasmodium*, of
which *Plasmodium vivax* and *Plasmodium falciparum* are most frequently involved in
human infections. The vector responsible for transmitting malaria to humans is the
Anopheles mosquito and only malaria infections caused by *Plasmodium falciparum*
may be fatal to humans.

After inoculation in the body, the sporozoites of Plasmodium begin to reproduce within
liver cells. Multiplication of *Plasmodium* sporozoites occurs by schizogony, in which a
single sporozoite can produce as many as 40, 000 merozoites. The invasion of
erythrocytes by hepatic merozoites begins the erythrocytic phase of malaria, causing
fever and other severe manifestations.

Erythrocytes infected with the malaria parasite *P. falciparum* disappear from the
peripheral circulation as they mature from the ring stage to trophozoites. This

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phenomenon is known as sequestration and results from parasitized erythrocyte
adherence to microvascular endothelial cells and erythrocytes in diverse organs. Severe
*Plasmodium falciparum*-malaria is characterized by excessive sequestration of
infected- and uninfected erythrocytes in the microvasculature of the affected organ.

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Thus, *Plasmodium falciparum* is an intracellular protozoan, which during its vertebrate
life cycle invades and multiplies in liver and red blood cells. The virulence of the
parasite is associated with the capacity of the infected erythrocyte to adhere to
endothelial cells and to erythrocytes, so called rosetting. This may cause impaired local
10  oxygen delivery and thereby death of the human host (Miller, L. H., F. Good, and G.
Milon. 1994. Malaria pathogenesis. *Science* 264, 1878-1883, Pasloske, B. L. and R.J.
Howard. 1994. Malaria, the red cell, and the endothelium. *Ann. Rev. Med.* 45, 283-295,
Marsh, K., M. English, J. Crawley, and N. Peshu, 1996. The pathogenesis of severe
malaria in African children. *Ann. Trop. Med. & Parasitol.* 90, 395-402). The most
15  malignant form of the infection is cerebral malaria, due to a massive sequestration of
infected and uninfected erythrocytes in the brain micro-vasculature.

After being transported from the internal parasite, antigens involved in the binding of
cells are thought to be concentrated and subsequently exposed to the exterior of the
20  erythrocyte at minute ($\approx$100 nm in diameter), electron-dense excrescence's called
knobs (Atkinson, C. T. and M. Aikawa, 1990. Ultrastructure of malaria-infected
erythrocytes. *Blood Cells* 16, 351-368 ). One such antigen is *Plasmodium falciparum*
erythrocyte membrane protein-1 (PfEMP1), a polypeptide of 200-350 kDa encoded by
the *var* family of *P. falciparum* genes (Howard, R. J., J.W. Barnwell, and V. Kao,
25  1983. Antigenic variation in *Plasmodium knowlesi* malaria: identification of the variant
antigen on infected erythrocytes. *Proc. Natl. Acad. Sci. U.S.A.* 80, 4129-4133, Su, X.-
Z, V.M. Heatwole, S.P. Wertheimer, F. Guinet, J.A. Herrfeldt, D.S. Peterson, J.A.
Ravetch, and T.E. Wellems. 1995. The large diverse gene family *var* encodes proteins
involved in cytoadherence and antigenic variation of *Plasmodium falciparum*-infected
30  erythrocytes. *Cell* 82, 89-100, Baruch, D. I., B.L. Pasloske, H.B. Singh, X. Bi, X.C.

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Ma, M. Feldman, T.F. Taraschi, and R.J. Howard. 1995. Cloning th *P. falciparum*
gene encoding PfEMP1, a malaria variant antigen and adherence receptor on the
surface of parasitized human erythrocytes. *Cell* 82, 77-87). The feature of antigenic
variation and switching of the surface of the pRBC has been attributed to the *var*-
genes. Even though up to 150 such genes are harboured in the genome, only one
PfEMP1 is thought to be expressed at any one time. PfEMP1 has features of an
adhesive molecule and has been associated with the cytoadherent properties of the
infected red cell (Smith, J. D., C.E. Chitnis, A.G. Craig, D.J. Roberts, D.E. Hudson-
Taylor, D.S. Peterson, R. Pinches, C.I. Newbold, and L.H. Miller. 1995. Switches in
expression of *Plasmodium falciparum var* genes correlate with changes in antigenic
and cytoadherent phenotypes of infected erythrocytes. *Cell* 82, 101-110, Baruch, D. I.,
J.A. Gormley, C. Ma, R.J. Howard, and B.L. Pasloske. 1996. *Plasmodium falciparum*
erythrocyte membrane protein 1 is a parasitised erythrocyte receptor for adherence to
CD36, thrombospondin, and intercellular adhesion molecule 1. *Proc. Natl. Acad. Sci.
U.S.A.* 93, 3497-3502). For example, the expression of PfEMP1-encoding *var* genes
has been shown to correlate with the capacity of the pRBC for binding to host
receptors, including CD36 and ICAM-1 (Baruch, D. I., J.A. Gormley, C. Ma, R.J.
Howard, and B.L. Pasloske. 1996. *Plasmodium falciparum* erythrocyte membrane
protein 1 is a parasitised erythrocyte receptor for adherence to CD36, thrombospondin,
and intercellular adhesion molecule 1. *Proc. Natl. Acad. Sci. U.S.A.* 93, 3497-3502,
Gardner, J.P., R.A. Pinches, D.J. Roberts, and C.I. Newbold, (1996). Variant antigens
and endothelial receptor adhesion in *Plasmodium falciparum. Proc. Natl. Acad. Sci.
U.S.A,* 93, 3503-3508). A role for PfEMP1 in rosetting was recently suggested by
Rowe *et al* (Rowe, J.A., J.M. Moulds, C.I. Newbold, and L.H. Miller. 1997. *P.
falciparum* rosetting mediated by a parasite-variant erythrocyte membrane protein and
complement-receptor 1. *Nature* 388:292-295 ). Complement receptor 1 was found to
be the host-receptor. However, it is beleived that it cannot be the only one used by
rosetting parasites.

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In WO 96/33736, one PfEMP1 variant is disclosed by sequence. However, this is only one of several possibly existing variants, all of which are different as to functional sequences as well as adhesive properties.

5     Accordingly, due to the complex nature and/or mechanism of malarial antigenic variation and the virulence thereof, there is still a great need for methods and compositions which may be useful in the treatment, diagnosis and prevention of malaria infections.

## Object of the invention

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The present invention fulfills the need described above by providing a carbohydrate, which is useful as a receptor for malaria erythrocyte membrane protein. The invention also provides such a new malaria protein acting as a ligand in relation to the receptors according to the invention.

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## Brief description of the drawings

Figure 1     discloses the identification of rosetting PfEMP1.

Figure 2A-C disclose, respectively, maps of cDNA structure, sequencing clones, deduced amino acid sequence and the location of GAG binding motifs in

20     the rosetting PfEMP1 of FCR3S1.2.

Figure 3     discloses the binding of rosetting FCR3S1.2- PfEMP1 to heparine as seen by gel analysis of 10% SDS-PAGE stained with Coomassie.

Figure 4     discloses the disruption of preformed natural rosettes with either one of the fusion proteins DBL-I-GST or ATS-GST.

25    Figure 5     discloses the effect of glycosaminoglycans on *Plasmodium falciparum* rosetting.

## Definitions

The term "glycosaminoglycan-like" as used in the present disclosure refers to any

30    molecule or substance, that exhibits the same or similar advantageous properties as the

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glycosaminoglycans comprising repeated units of sulfated or acetylated disaccharides and further discussed below.

As used herein, the term "essentially specific binding" in the context of the carbohydrates according to the invention is interpreted as such a specific binding as is needed to influence the rosetting of malaria erythrocyte membrane protein without having any other notable impact on the environment.

The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers, in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The terms "isolated" "purified" or "biologically pure" refer to material which is substantially or essentially free from components which normally accompany it as found in its native state.

The term "biologically active fragment" as used herein refers to portions of the proteins or polypeptides possessing a particular biological activity, e.g. one or more activities found in the full length novel PfEMP1 variant according to the invention.

The term "fusion protein" as used herein generally refers to a composite protein made up of two or more separate, heterologous proteins.

The term "substantial identity" or "substantial similarity" in the context of a polypeptide indicates that a polypeptides comprises a sequence with at least 70% sequence identity to a reference sequence, or preferably 80%, or more preferably 85% sequence identity to the reference sequence, or most preferably 90% identity over a comparison window of about 10-20 amino acid residues. An indication that two polypeptide sequences are substantially identical is that one peptide is immunologically reactive with antibodies raised against the

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second peptide. Thus, a polypeptide is substantially identical to a second polypeptide, for example, where the two peptides differ only by a conservative substitution.

A "conservative substitution", when describing a protein, refers to a change in the amino acid composition of the protein that does not substantially alter the protein's activity. Thus, "conservatively modified variations" of a particular amino acid sequence refers to amino acid substitutions of those amino acids that are not critical for protein activity or substitution of amino acids with other amino acids having similar properties (*e.g.*, acidic, basic, positively or negatively charged, polar or non-polar, *etc.*) such that the substitutions of even critical amino acids do not substantially alter activity. Conservative substitution tables providing functionally similar amino acids are well known in the art. ( See, *e.g.*, Creighton (1984) *Protein*, W.H. Freeman and Company.) In addition, individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids in an encoded sequence are also "conservatively modified variations".

An indication that two nucleic acid sequences are substantially identical is that the polypeptide which the first nucleic acid encodes is immunologically cross reactive with the polypeptide encoded by the second nucleic acid.

Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions, as discussed in more detail below.

The term "nucleic acid" refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, encompasses known analogues of natural nucleotides that can function in a similar manner as naturally occurring nucleotides.

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The phrase "hybridizing specifically to", refers to the binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a complex mixture (*e.g.*, total cellular) DNA or RNA. The term "stringent conditions" refers to conditions under which a probe will hybridize to its

5    target subsequence, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point Tm for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength, pH, and

10    nucleic acid concentration) at which 50% of the probes complementary to the target sequence hybridize to the target sequence at equilibrium. (As the target sequences are generally present in excess, at Tm, 50% of the probes are occupied at equilibrium). Typically, stringent conditions will be those in which the salt concentration is less than about 1.0 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at

15    pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (*e.g.*, 10 to 50 nucleotides) and at least about 60°C for long probes (*e.g.*, greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents, such as formamide.

20    As used herein a "nucleic acid probe" is defined as a nucleic acid capable of binding to a target nucleic acid of complementary sequence through one or more types of chemical bonds, usually through complementary base pairing, usually through hydrogen bond formation. As used herein, a probe may include natural (*i.e.* A, G, C, or T) or modified bases (7-deazaguanosine, inosine, *etc.*) In addition, the bases in a probe may be joined by

25    a linkage other than a phosphodiester bond, so long as it does not interfere with hybridization. Thus, for example, probes may be peptide nucleic acids in which the constituent bases are join d by peptide bonds rather that phosphodiester linkages. It will be understood by one of skill in the art that probes may bind target sequences lacking complete complementarity with the probe sequence depending upon the stringency of the

30    hybridization conditions. The probes are preferably directly labelled as with isotopes,

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chromophores, lumiphore, chromogens, or indirectly labelled such as with biotin to which a streptavidin complex may later bind. By assaying for the presence or absence of the probe, one can detect the presence or absence of the select sequence or subsequence.

5      A "label" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include $^{32}$P, fluorescent dyes, electron-dense reagents, enzymes (e.g., as commonly used in a ELISA), biotin, dioxigenin, or haptens and proteins for which antisera or monoclonal antibodies are available (e.g., the peptide of the invention can be made detectable, e.g., by incorporating

10     a radio-label into the peptide, and used to detect antibodies specifically reactive with the peptide).

A "labelled nucleic acid probe" is a nucleic acid probe that is bound, either covalently, through a linker, or through ionic, van der Waals or hydrogen bonds to a label such that

15     the presence of the probe may be detected by detecting the presence of the label bound to the probe.

The term "target nucleic acid" refers to a nucleic acid (often derived from a biological sample), to which a nucleic acid probe is designed to specifically hybridize. It is either the

20     presence or absence of the target nucleic acid that is to be detected, or the amount of the target nucleic acid that is to be quantified. The target nucleic acid has a sequence that is complementary to the nucleic acid sequence of the corresponding probe directed to the target. The term target nucleic acid may refer to the specific subsequence of a larger nucleic acid to which the probe is directed or to the ovarall sequence (e.g., gene or

25     mRNA) whose expression level it is desired to detect. The difference in usage will be apparent from context.

"Bind(s) substantially" refers to complementary hybridization between a probe nucleic acid and a target nucleic acid and embraces minor mismatches that can be acc modated

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by reducing the stringency of the hybridization media to achieve the desired detection of the target polynucleotide sequence.

"Subsequence" refers to a sequence of nucleic acids or amino acids that comprise a part
5    of a longer sequence of nucleic acids or amino acids (e.g., polypeptide), respectively.

In the present disclosure, the term "antibody" refers to a polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof which specifically bind and recognize an analyte (antigen). The recognized
10   immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

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A "chimeric antibody" is an antibody molecule in which (a) the constant region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable region) is linked to a constant region of a different or altered class, effector function and/or species, or an entirely different molecule which confers new properties to the
20   chimeric antibody, e.g., an enzyme, toxin, hormone, growth factor, drug, etc.; or (b) the variable region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity.

The term "immunoassay" is an assay that utilizes an antibody to specifically bind an
25   analyte. The immunoassay is characterized by the use of specific binding properties of a particular antibody to isolate, target, and/or quantify the analyte.

The phrases "specifically binds to a protein" or "specifically immunoreactive with", when referring to an antibody refers to a binding reaction which is determinative of the
30   presence of the protein in the presence of a heterogeneous population of proteins and

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other biologics. Thus, under designated immunoassay conditions, the specified antibodies
bind preferentially to a particular protein and do not bind in a significant amount to other
proteins present in the sample. Specific binding to a protein under such conditions
requires an antibody that is selected for its specificity for a particular protein. A variety of
5     immunoassay formats may be used to select antibodies specifically immunoreactive with
a particular protein. For example, solid-phase ELISA immunoassays are routinely used to
select monoclonal antibodies specifically immunoreactive with a protein. See Harlow and
Lane (1988) *Antibodies, A Laboratory Manual,* Cold Spring Harbour Publications, New
York, for a description of immunoassay formats and conditions that can be used to
10    determine specific immunoreactivity.

The term "recombinant" when used with reference to a cell, or nucleic acid, or vector,
indicates that the cell, or nucleic acid, or vector, has been modified by the introduction of
a heterologous nucleic acid or the alteration of a native nucleic acid, or that the cell is
15    derived from a cell so modified. Thus, for example, recombinant cells express genes that
are not found within the native (non-recombinant) form of the cell or express native genes
that are otherwise abnormally expressed, under expressed or not expressed at all. The
term "identical" in the context of two nucleic acids or polypeptide sequences refers to the
residues in the two sequences which are the same when aligned for maximum
20    correspondence. Optimal alignment of sequences for comparison can be conducted, *e.g.,*
by the local homology algorithm of Smith and Waterman (1981) *Adv. Appl. Math.* 2: 482,
by the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.*
48:443, by the search for similarity method of Pearson and Lipman (1988) *Proc. Natl.*
*Acad. Sci.* USA 85: 2444, by computerized implementations of these algorithms (GAP,
25    GESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics
Computer Group, 575 Science Dr., Madison, WI) or by inspection. An additional
algorithm that is suitable for determining sequence similarity is the BLAST algorithm,
which is described in Altschul *et al.* (1990) *J. Mol. Biol.* 215: 403-410. Software for
performing BLAST analyses is publicly available through the National Center for
30    Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

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## Detailed description of the present invention

The present invention is disclosed in more detail below. All references throughout the present application are hereby included herein by references.

Thus, in a first aspect, the present invention relates to a carbohydrate, which exhibits at least one negatively charged glycosaminoglycan-like moiety and thereby is capable of essentially specific binding to a malaria erythrocyte membrane protein or an analogue thereof. Accordingly, the carbohydrate according to the invention may be used as a receptor to bind ligands, which are expressed on the surface of cells infected by malaria, such as erythrocytes infected by *Plasmodium falciparum*. In a preferred embodiment, said glycosaminoglycan moiety is sulfated and more specifically, it may be a heparan sulfate moiety. Most preferably, the carbohydrate according to the invention is fucoidan, or a functional fragment thereof. Further, the invention also relates to any functionally equivalent analogues of the above described carbohydrates.

Indeed, it has been shown in the prior art that *P. falciparum*-rosettes may be disrupted by low concentrations of heparin, an effect that is immediate and reversible (Carlson, J., H.P. Ekre, H. Helmby, J. Gysin, B.M. Greenwood, and M. Wahlgren. 1992. Disruption of *Plasmodium falciparum* erythrocyte rosettes by standard heparin and heparin devoid of anticoagulant activity. *Am. J. Trop. Med. Hyg.* 46, 595-602, Rowe, A., A.R. Berendt, K. Marsh, and C.I. Newbold. 1994. *Plasmodium falciparum*: a family of sulfated glycoconjugates disrupts erythrocyte rosettes. *Exp. Parasitol.* 79, 506-16). However, prior to the present invention, it has not been possible to explain the mechanism or basis for this phenomena and, accordingy, it has not been possible to create any such advantageous and useful receptors as the present receptor carbohydrate until now. Thus, for the first time, the present invention discloses receptors capable of essentially specific binding of such malaria proteins.

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The glycosaminoglycans, or GAGs, being the receptor carbohydrate according to the invention, are composed of repeated units of sulfated or acetylated di-saccharides and are present in the human body bound to a protein-core in the form of proteoglycans (PG) (Yanagisha, M. and V. Hascall. 1992. Cell surface heparan sulfate proteoglycans.

5    J. Biol. Chem. 267, 9451-9454). Heparan sulfate and chondroitin sulfate are GAGs exposed at all human cell-surfaces, but they are diverse and vary from cell-to-cell and maybe within the same cell due to secondary modifications of the extensive carbohydrate chains (deacetylations, O-sulfations etc.). Heparin is only found in mast-cells and is characterized by a higher degree of sulfation and epimerization than

10    heparan sulfate. However, heparan sulfate and heparin are similar due to heparin-like stretches also found in heparan sulfate (Faham,S., E.R. Hileman, R.J. Fromm, J.R. Lindardt, and C.D. Rees. 1996. Heparin structure and interactions with basic fibroblast growth factor. Science, 271, 1116-1120 ).

15    HS and heparin, both members of the heterogeneous GAG family, are composed of alternating glucosamine and uronic acid residues. The glucosamine can be either N-sulfated or N-acetylated and can contain an O-sulfate ester at C-6. The uronic acid exists as either a glucoronic or an iduronic acid epimer and may be O-sulfated at C-2. In contrast to heparin chains which are extensively sulfated, HS chains are less

20    extensively modified and the sulfate groups are unevenly distributed over the chains due to incompleteness of modification in each step of HS biosynthesis (Lindahl et al. 1994) Consequently, HS and heparin are similar due to highly sulfated, heparin-like stretches found in HS.

25    In general, micro-heterogeneity within the structure of GAGs modulates their binding and biological activities (Casu 1991). Their binding properties are usually associated with their anionic sulfate and carboxyl groups. Increasing charge density and mol cular weight is often associated with stronger protein binding, but this is not a strict rule (Ruoslahti 1989). Thus, a general increase in binding properties can be achieved by

30    chemical oversulfation (Casu 1991). Most GAGs express their biological properti s

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through interactions with plasma and tissue components (Jackson *et al.* 1991; Kjellén *et al.* 1991) and their ubiquitous distribution on cell surfaces make them well suited for microbial attachment.

5      In a particular embodiment, the receptor carbohydrate, or said functional equivalent thereof, is more specifically capable of essentially specific binding to at least one of the segments denoted binding sites of the amino acid sequence according to SEQ ID NO:1, wherein a novel variant of Plasmodium falciparum erythrocyte membrane protein (PfEMP1) is disclosed. Even though it is more fully discussed elsewhere in this
10     application, especially in connection with the ligand polypeptide below, it should be understood that said binding segments are sequences, which, according to the present invention, have been found to bind specifically and strongly to glycosaminoglycan-like moieties on receptor substances, such as the ones present on most cells or the present polypeptide. Thus, in a specific embodiment, the receptor carbohydrate according to
15     the invention is capable of binding to an amino terminal part, and preferably an essential part, of the binding segments indicated in SEQ ID NO:1. Consequently, the protein disclosed in SEQ ID NO:1 may be regarded as a ligand, whereas the carbohydrate defined as above may be regarded as the receptor therefore. Most preferably, the receptor binds to an essential part of said sequence or any sequence
20     having a substantial identity or similarity with SEQ ID NO:1.

The carbohydrate receptor according to the present invention may be prepared by fractionation from animal cells or by conventional methods, which are well known in the art (see *e.g.* Binkley: *Modern Carbohydrate Chemistry*, Marcel Dekker, New York,
25     1988, with references; and US patents Nos. 5 308 460 and 5 532 147).

In another aspect, the present invention relates to a carbohydrate as defined above for use as a medicament as well as the use of said receptor as a medicament. Such a medicament is effective in that it is capable of effectively dissolving the rosettes
30     formed by erythrocytes infected by a malaria parasite, as described more detailed in the

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introductory section of this application. Thereby, a medicament comprising the receptor carbohydrate according to the invention in a suitable form will be effective in dissolving and preventing the occlusion of blood vessels, especially in cerebral malaria. Accordingly, the invention also encompasses the use of said carbohydrate in

5    the manufacture of a medicament against malaria, preferably against *P. falciparum* malaria. Thus, in one embodiment, the invention relates to a pharmaceutical composition comprising a carbohydrate according to the invention in a pharmaceutically or veterinary acceptable carrier. Other additives or excipients which are deemed suitable for the specific application may also be present in such a

10   composition. Further, the present invention also relates to any other substance, which has been achieved by use of parts or all of the novel polypeptide defined in SEQ ID NO:1, or, alternatively, by use of a carbohydrate according to the invention as a lead substance, for use as medicaments, for use in the manufacture of pharmaceutical preparations and pharmaceutical compositions comprising such a substance together

15   with a carrier. Methods for producing such substances, which are functionally equivalent to the present carbohydrates, will be discussed in more detail below.

One more aspect relating to the above defined carbohydrate, or malaria protein receptor substance, is a method of treating a patient suffering from a malaria infection,

20   preferably a *P. falciparum* infection. Such a method comprises administering to the patient of an effective amount of the pharmaceutical composition described in detail elsewhere in the present description.

Another aspect of the present invention is a polypeptide, which is capable of acting as a

25   ligand in relation to the receptor carbohydrate described above. Said ligand polypeptide originates from a malaria erythrocyte membrane protein and may be denoted a novel PfEMP1-variant.

Thus, by single-cell RT-PCR, the present inventor has identified a novel PfEMP1-

30   variant of a rosetting parasite. Clusters of GAG-binding motifs have been identified in

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the sequence, denoted binding sites in the sequence listing below. In addition, it has been shown that the recombinant form of the novel PfEMP1-variant, or ligand polypeptide, according to the invention, adheres to solid-phase heparin, to heparan sulfate on the erythrocyte surface, and disrupts rosettes. Further, naturally formed rosettes also seem to be mediated by binding to the same GAG.

In an advantageous embodiment, the ligand polypeptide according to the invention comprises at least about 300 amino acids of the amino acid sequence according to SEQ ID NO:1. Preferably, the ligand comprises an amino terminal sequence of said sequence, or an analogue thereof. More preferably, the ligand according to the invention comprises about 400-500 amino acids, and most preferably about 400 amino acids (DBL-1), such as about 423 amino acids, thereof. In addition, the ligand polypeptide according to the invention is capable of essentially specific binding to a negatively charged glycosaminoglycan-like moiety. In the preferred embodiment thereof, the ligand polypeptide according to the invention is capable of binding any receptor carbohydrate according to the invention and described above. In a particular embodiment thereof, the polypeptide according to the invention comprises essentially all of the sequence according to SEQ ID NO:1, wherein the complete amino acid sequence of FCR3S1.2 PfEMP1 is shown. The location of potential GAG binding motifs are shown in pink. Motifs no.4, 5 and 9,10 (aa 221-232 and 533-549, respectively) are seen as a single stretch as they are located next to each other (see methods section for description of identification of GAG-binding motifs). These sequence data are available from GenBank under accession number of AF003473.

The weight of the polypeptide according to the invention will depend on the length of the amino acid sequence as discussed above but may be about 100-300 kDa, preferably about 280 kDa. Further, the present invention also relates to any biologically active fragments of the herein disclosed polypeptides and proteins.

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Another aspect of the invention is a method of pr paring a ligand polypeptide as defined herein, which comprises the steps of

(1) the inserting into an expression vector of a nucleic acid encoding said ligand polypeptide or functionally equivalent analogue thereof;

5 (2) the transfection of a host cell capable of expressing said nucleic acid with    said expression vector to express said polypeptide; and

(3) the recovery of the expressed polypeptide and advantageously purification thereof to a biologically pure form. Expression vectors, host cells, process parameters *etc.* are easily chosen by someone skilled in this area.

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Accordingly, a further aspect of the invention is vectors on host cells useful in the above disclosed method and comprising nucleic acids encoding a polypeptide according to the invention.

15 In one particular embodiment of the invention, the polypeptide may be cloned using DNA amplification methods, such as the polymerase chain method (PCR) (*see e.g.* Sambrook *et al., Molecular Cloning: A Laboratory Manual,* 2nd ed., Cold Spring Harbour, N.Y., 1989; Berger & Kimmel, *Methods in Enzymology,* Vol. 152: *Guide to Molecular Cloning Techniques,* Academic Press, Inc., San Diego, Calif., 1987; Co *et al* 
20 *(1992) J. Immunol.* 148:1149). Thus, for example, the nucleic acid sequence, which will be disclosed in more detail below, or subsequence is PCR amplified using a sense primer containing one restriction site and an antisense primer containing another restriction site. This will produce a nucleic acid encoding the desired sequence or subsequence having terminal restriction sites. This nucleic acid can then easily be 
25 ligated into a vector having appropriate corresponding restriction sites. Suitable PCR primers are easily chosen by one of skill in the art based on the sequence to be expressed. Appropriate restriction sites can also be added by site-directed mutagenesis (*see* Gillman & Smith (1979) *Gene,* 8: 81-97; Roberts *et al.* (1987) *Nature* 328: 731-734).

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The nucleic acids according to the invention, which are described in more detail below, may be expressed in a variety of host cells, including *E. coli*, other bacterial hosts, yeast and various higher eucaryotic cells, such as the COS, CHO and HeLa cell lines and myeloma cell lines. The recombinant protein gene will be operably linked to

5    appropriate expression control sequences for each host. For *E.coli*, this includes a promoter, such as the T7, trp, or lambda promoters, a ribosome binding site and preferably a transcription termination signal. For eucaryotic cells, the control sequences will include a promoter and preferably an enhancer derived from immunoglobulin genes, SV40, cytomegalovirus *etc.*, and a polyadenylation sequence,

10    and may include splice donor and acceptor sequences. The plasmids can be transferred into the chosen host cell by well-known methods, such as calcium chloride transformation for *E. coli* and calcium phosphate treatment or electroporation for mammalian cells. Cells transformed by the plasmids can be selected by resistance to antibiotics conferred by genes contained on the plasmids, such as amp, gpt, neo and

15    hyg genes.

Once expressed, the recombinant polypeptides according to the invention may be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel electrophoresis and the

20    like (*see* generally R. Scopes, *Protein Purification*, Springer-Verlag, N.Y. (1982), Deutcher, *Methods in Enzymology vol. 182: Guide to Protein Purification*, Academic Press, Inc. N.Y. (1990)). Substantially pure compositions are preferred, such as at least about 90-95% homogeneity, and most preferred, 98-99% homogeneity. Once purified, partially or to the homogeneity as desired, the polypeptides according to the invention

25    may be used, *e.g.* as immunogens for antibodyproduction.

As one of skill in this field would recognize, after chemical synthesis, biological expression, or purification, the proteins, polypeptides or fusion proteins according to the present invention may possess a conformation substantially different from the

30    native conformations of the constituent polypeptides. In this case, it may be necessary

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to denature and reduce the polypeptide and then to cause the polypeptide to re-fold into the preferred conformation. Methods of reducing and denaturing proteins and inducing re-folding are well known to those of skill in the art (*see e.g.* Debinski *et al.* (1993) *J. Biol. Chem.*, 268: 14065-14070; Kreitman and Pastan (1993) *Bioconj. Chem.*, 4:581-585; and Buchner *et al.* (1992) *Anal. Biochem.*, 205:263-270).

One of skill would recognize that modifications can be made to the present polypeptides and proteins without diminishing their biological activity, e.g. in order to facilitate cloning, expression or incorporation of the targeting molecule into a fusion protein. Such modifications are well known and any thus modified peptides and proteins are understood to be encompassed within the scope of the present invention as defined by the appended claims.

Further, the present invention also relates to vectors and recombinant host cells comprising the sequence according to SEQ ID NO:1 or any biologically active fragment thereof. In addition, the invention also encompasses any isolated cell comprising the sequence according to SEQ ID NO:1 as an endogenous gene, which has been manipulated by gene activation, that is, wherein additional regulatory sequences have been introduced in order to increase the expression of the native coding sequence (see e.g. US patent no. 5 578 461 and US patent no. 5 641 670). The culture of cells according to the present invention is well known in the art (see e.g. Freshney, Culture of Animal Cells, A Manual of Basic Technique, third ed., Wiley-Liss, New York (1994) and the references cited therein for a general guide to the culture of cells).

As indicated above, the present invention also relates to nucleic acids encoding the polypeptides according to the invention as well as to any nucleic acid capable of specific hybridization, under stringent conditions, to such a nucleic acid. The nucleic acids according to the invention thus include, but are not limited to, DNAs, RNAs, an mRNA transcript, a cDNA reverse transcribed from a mRNA, and RNA transcribed from that cDNA, a DNA amplified from the cDNA, an RNA transcribed from the

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amplified DNA or subsequences of any of these nucleic acids and may be prepared by

any suitable method, which is easily chosen by someone skilled in this field. Such

methods include, for example, cloning and restriction of appropriate sequences or

direct chemical synthesis by methods such as the phosphotriester method (Narang et

5    al., *Meth. Enzymol.* 68:90-99 (1979)); the phosphodiester method (Brown *et al., Meth.

*Enzymol.* 68: 109-151 (1979)); the diethylphpsphoramidite method (Beaucage *et al.,*

*Tetra. Lett.*, 22:1859-1862 (1981)); and the solid support method (US Patent no. 4 458

066).

10    Chemical synthesis produces a single stranded oligonucleotide. This may be converted

into double stranded DNA by hybridization with a complementary sequence or by

polymerisation with a DNA polymerase using the single strand as a template.The DNA

sequence obtained by chemical synthesis is limited in length by the technique used,

however, longer sequences are easily obtained by the ligation of such shorter

15    sequences to the desired length. Alternatively, subsequences may be cloned and the

appropriate subsequences cleaved using suitiable restriction enzymes. The fragments

may then be ligated to produce DNA sequences of the desired size.

A nucleic acid according to the invention may *e.g.* be used in the above method for

20    preparing a polypeptide, as a probe *etc*. For the use thereof as a probe, it is often

desirable to label the sequence with a detectable label. The label may be incorporated

during the amplification step in the preparation of the nucleic acid, e.g. by PCR, by

transcription amplification using a labelled nucleotide incorporationg a label in the

transcribed nucleic acid or, alternatively, by direct addition thereof, such as by nick-

25    translation or any other suitable method. Detectable labels suitable for use according to

the present invention include any composition detectable by spectroscopic,

photochemical,biochemical, immunochemical, electrical, optical or chemical means

and methods for the detection thereof are well known to those of skill in the art.

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In an alternative aspect of the invention, the polypeptide is produced in the form of a recombinant fusion protein, which comprises said polypeptide, comprising suitable glycosaminglycan-like moieties, fused to another protein or polypeptide. Such a polypeptide comprising fusion protein may be more advantageous for certain

5      applications than the polypeptide *per se*. In a preferred embodiment of the invention, the fusion proteins are synthesized using recombinant nucleic acid methodology. Generally, this involves creating a nucleic acid that encodes the receptor-targeted fusion molecule, placing the nucleic acid in an expression cassette under the control of a suitable promoter, expressing the protein in a host, isolating the expressed protein

10     and, if required, renaturing the protein. Techniques sufficient to guide one of skill through such procedures are found in *e.g.*, Sambrook *et al.*, *Molecular Cloning. A Laboratory Manual*, $2^{nd}$ ed., vol. 1-3, Cold Spring Harbor Laboratory, 1989; Berger and Kimmel, *Guide to Molecular Cloning Techniques*, Methods in Enzymology, vol. 152, Academic Press, Inc., San Diego, CA; and *Current Protocols in Molecular*

15     *Biology*, F.M. Ausubel *et al.*, eds., Current Protocols (Greene Publishing Associates , Inc. And John Wiley & Sons, Inc.) (1994 Supplement). Alternatively, the fusion protein is produced by any method for chemical synthesis well known in the art. Thus, a fusion protein according to the invention may comprise any polypeptide, antibody or biologically active fragment thereof according to the present invention. The effects of

20     exemplary fusion proteins on rosetting is e.g. discussed in the Experimental section of this specification, see *e.g.* Fig 4.


The present invention also relates to a polypeptide according to the invention for use as a medicament as well as the use thereof in the manufacture of a medicament. Such a

25     medicament is effective in that it is capable of effectively dissolving the rosettes formed by erythrocytes infected by a malaria parasite, as described more detailed in the introductory section of this application. Thereby, a medicament comprising the polypeptide according to the invention, *per se* or in the form of a fusion protein, will be effective in dissolving and preventing the occlusion of blood vessels, esecially in

30     cerebral malaria. In addition, the above described rosettes have also been shown to be

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associated with other severe complications of malaria (Carlson, J., et al, Lancet 336, 1457-1460 (1990); Treutiger et al, Am. J. Trop. Med. Hyg. 46, 503-510 (1992); and Rowe, A et al, Inf. Immun 63, 2323-2326 (1995)). Thus, a medicament comprising a polypeptide according to the invention is also effective in the treatment of such other

5    conditions. Accordingly, the invention also encompasses the use of said polypeptide in the manufacture of a medicament against malaria, preferably *P. falciparum* malaria, and/or other severe complications of malaria.Consequently, in a further aspect, the invention relates to a pharmaceutical preparation comprising said polypeptide or fusion protein in a pharmaceutically or veterinary acceptable carrier, e.g. an aqueous carrier,

10   such as buffered saline or the like. These solutions are sterile and generally free of any undesirable matter. They may be sterilized by conventional, well known sterilization techniques. The pharmaceutical preparations according to the invention may be used in any suitable method of administration, such as in parenteral, topical, oral or even local administration for prophylactic and/or therapeutic treatment and may be administered

15   in a variety of unit dosage forms depending on the administration method chosen. Further, the pharmaceutical compositions according to the invention may comprise any pharmaceutically acceptable auxiliary substances required to approximate physiological conditions, such as pH adjusting and buffering agents, toxicity adjusting agents and the like, for example sodium acetate, sodium chloride, potassium chloride,

20   calcium chloride, sodium lactate *etc*. Actual methods for preparing the appropriate administrable compositions will be known or apparent to those skilled in this field and are described in more detail in *e.g. Remington's Pharmaceutical Science*, 15th ed., Mack Publishing Company, Easton, Pennsylvania (1980). (For a brief review of methods for drug delivery, see Langer, Science 249:1527-1533 1990)).

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Accordingly, another aspect of the present invention is a method of treating a patient suffering from a malaria infection by the administration of the present polypeptide or a fusion protein or, alternatively, preventing malaria in a subject at risk of being infected thereof. It is to be understood, that in the present context, the term "preventing" refers

30   to the prevention of a disorder, such as malaria, or the symptoms thereof, by use of a

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medicament as well as such a prevention by use of a vaccine composition. Thus, the present invention encompass preventive or prophylactic treatments and medicaments used therein, which treatments will include drugs that actively participate in the prevention of a particular disorder, such as malaria or the symptoms thereof. Further,

5    the invention also encompases treatments that elicit a preventive response from a patient, such as an immunological response in the case of vaccination. Consequently, "a medicament for the prevention of malaria" includes also includes vaccine compositions. The method comprises administering to said patient of an effective amount of the herein described pharmaceutical composition, said amount being

10   dependent on the severity of the disease and the general state of the health of the patient. Preferably, the malaria infection treated by the method according to the invention is a *P. falciparum* infection.

In the preferred embodiment, the vaccine compositions according to the invention will

15   include a molecule according to the invention, such as a polypeptide, in an immunologically effective amount together with a suitable pharmaceutically acceptable carrier. In this context, examples of suitable carriers are e.g. thyroglobulin, albumins, such as human serum albumin, tetanus toxoid, polyamino acids, such as poly(D-lysine; D-glutamic acid), influenza, hepatitis B virus core protein as well as other carriers well

20   known to those skilled in the art. The vaccines may also include a physiologically tolerable diluent, such as water, buffered water, buffered saline, saline and they may further also include any suitable adjuvant, such as incomplete Freunds' adjuvant, aluminium phosphate, aluminium hydroxide, alum, ammonium hydroxide in case of human pateient, etc. The immune response of the patient may include generation of

25   antibodies, activation of cytotoxic T-lymphocytes against cells expressing the polypeptides or other mechanisms known within this field. (See *e.g.* Paul, *Fundamental Immunology*, 2nd ed., Raven Press; Sedagh et al., *Proc. Natl. Acad. Sci.* (1994) 91:9866-9877; US patent no. 4 722 848; and Langford, C.L. et al., 1986, *Mol. Cell. Biol.* 6:3191-3199.)

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Another highly interesting and advantageous aspect of th  present invention is the use of the polypeptide as defined above as a model substance for identifying substances binding to a malaria erythrocyte membrane protein, such as the novel variant disclosed in SEQ ID NO:1, or analogues thereof. Thereby, new receptor substances effective in

5     the treatment and/or prevention of malaria infections may be isolated from suitable environments or bulks comprising the same.

More specifically, a polypeptide according to the invention, or a fragment thereof, is used in the design of an organic compound, which is modeled to resemble the three

10    dimensional structure of the amino acid residues of said polypeptide. Such a design of mimetics to a known pharmaceutically active compound is a known approach to the development of pharmaceuticals based on a "lead" compound. Mimetic design, synthesis and testing is generally used to avoid randomly screening large number of molecules for a target property. In relation to mimetics, or mimics, as they are

15    sometimes denoted, see for example Shikman A.R. and Cunningham M.W., *J. Immunol.* 152(9):4375 (1994); Vaughan *et al., Xenotransplantation* 3:18-23 (1996); and Koogman *et al*, Dean (1994), BioEssays, 683-687; Cohen and Shatzmiller (1993), *J. Mol. Graph.*, 11: 166-173; Wiley and Rich (1993), *Med. Res. Rev.*, 13:327-384; Moore (1994), *Trends Pharmacol. Sci.*, 15:124-129; Hruby (1993), *Biopolymers*, 33:

20    1073-1082; Bugg et al. (1993), Sci. Am., 269:92-98.

In brief, the particular parts of the compound that are critical and/or important are firstly determined. This can be done systematically by varying the amino acid residues in the peptide, e.g. by substituting each residue in turn. These parts or residues

25    constituting the active region of the compound are known as its "pharmacophore". Once the pharmacophore has been found, its structure is modelled according to its physical properties, e.g. stereochemistry, bonding, size and/or charge, using data from a range of sources, e.g. spectroscopic techniques, X-ray diffraction data and NMR. Computational analysis, similarity mapping (which models the charge and/or volume

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of a pharmacophore, rather than the bonding between atoms) and other techniques can be used in this modelling process.

In a specific embodiment, the three dimensional structure of the ligand and its binding partner are modelled. This can be especially useful where the ligand and/or binding partner (receptor) change conformation on binding, allowing the model to take account of this in the design of the mimetic.

A template molecule is then selected onto which chemical groups which mimic the pharmacophore can be grafted. The template molecule and the chemical groups grafted onto it can conveniently be selected so that the mimetic is easy to synthesize, is likely to be pharmacologically acceptable and does not degrade *in vivo*, while retaining the biological activity of the lead compound. The mimetic(s) found by this approach can then be screened to see whether or not they have the target property, or to what extent they exhibit it. Further optimisation or modification can then be carried out to arrive at one or more final mimetics for *in vivo* or clinical testing. Accordingly, the use of the present polypeptide ligand and/or carbohydrate binding partner as models to identify optimal pharmaceutically active substances, preferably for preventing and/or treating malaria, is encompassed by the present invention as are any substances identified thereby, which fulfills the herein desired criteria.

In another aspect, the invention relates to diagnostic and screening assays, wherein *e.g.* the polypeptides according to the invention are used for the detection of antibodies or wherein antibodies as disclosed below are used to detect PfEMP1 or fragments thereof. Further, nucleic acids may be detected in biological samples. (For a review of diagnostic immunoassay procedures, see *e.g. Basic and Clinical Immunology*, 7th ed., Stites, D., and Terr, A., 1991.)

One further aspect of the invention is an antibody, which is specifically immunoreactive with a ligand polypeptide according to the present invention. Such an

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antibody may be used to formulate another pharmaceutical composition together with suitable pharmaceutically and/or veterinary acceptable carriers, excipients etc. Accordingly, the present invention also relates to such antibody compositions as well as to methods of treating and/or preventing malaria infection in a patient. Such

5     methods comprises administering to said patient of an effective amount of the pharmaceutical composition defined above. Most preferably, the infection to be treated and/or prevented is a *P. falciparum* infection.

Thus, antibodies are raised to the polypeptides of the present invention, including

10    individual, ellelic, strain, or species variants, and fragments thereof, both in their naturally occurring (full-length) forms and in recombinant forms. Additionally, antibodies are raised to these polypeptides in either their native configuarions or in non-native configurations. Anti-idiotypic antibodies can also be generated. Many methods of making antibodies are known to persons of skill. (In this context, see *e.g. Fundamental*

15    *Immunology*, Third Edition, W.E. Paul, ed., Raven Press, N.Y. 1993.) While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies or

20    those synthesized *de novo* using recombinant DNA methodologies (*e.g.*, single chain Fv).

A number of immunogens are used to produce antibodies specifically reactive with polypeptides according to the invention. Recombinant or synthetic polypeptides according to the invention of 8-15, preferably 10, amino acids in length, or greater, are

25    the preferred polypeptide immunogen (antigen) for the production of monoclonal or polyclonal antibodies.

Methods of producing polyclonal antibodies are known to those of skill in the art. See, *e.g.*, Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; and Harlow

30    and Lan  (1989) *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY.

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Monoclonal antibodies are prepared from cells secreting the desired antibody. These antibodies are screened for binding to normal or modified polypeptides, or screened for agonistic or antagonistic activity, *e.g.*, activity mediated through a *suppressor of fused* protein. Specific monoclonal and polyclonal antibodies will usually bind with a $K_D$ of at least about 0.1 mM, more usually at least about 50 µM, and most preferably at least about 1 µM or better.

In some instances, it is desirable to prepare monoclonal antibodies from various mammalian hosts, such as mice, rodents, primates, humans, *etc.* Description of techniques for preparing such monoclonal antibodies are found in, *e.g.* Stites *et al.* (eds) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therin; Harlow and Lane, *supra*; Goding (1986) *Monoclonal Antiboides: Principles and Practice* (2nd ed.) Academic Press, New York, NY; and Kohler and Milstein (1975) *Nature* 256: 495-497.

Other suitable techniques involve selection of libraries of recombinant antibodies in phage or similar vectors (*see, e.g.,* Huse *et al.* (1989) *Science* 246: 1275-1281; and Ward, *et al.* (1989) *Nature* 341: 544-546; and Vaughan *et al.* (1996) *Nature Biotechnology*, 14: 309-314).

Advantageously, the polypetides and antibodies according to the invention are labelled by joining, either covalently or non-covalently, a substance which provides for a detectable signal. A wide variety of labels, and conjugation techniques are known and are reported extensively in both the scientific and patent literature. See for example patents teaching the use of such labels, including U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

In a specific embodiment of the invention, the present antibodies are used for affinity chromatography in isolating polypeptides. Columns are prepared, *e.g.*, with the antibodies

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linked to a solid support, *e.g.*, particles such as agarose, Sephadex, or the like, where a cell lysate is passed through the column, washed, and treated with increasing concentrations of a mild denaturant, whereby purified polypeptides are released.

5    The antibodies can be used to screen expression libraries for particular expression products. Usually the antibodies in such a procedure are labelled with a moiety allowing easy detection of presence of antigen by antibody binding.

Antibodies raised against the present polypeptides can also be used to raise antiidiotypic

10    antibodies. These are useful for detecting or diagnosing the various pathological conditions related to the presence of the respective antigens.

The antibodies of this invention can also be administered to an organism (*e.g.*, a human patient) for therapeutic purposes. A large numer of methods of generating chimeric

15    antibodies are well known to those of skill in the art (*see*, *e.g.*, U.S. Patent Nos: 5,502,167, 5,500,362, 4,491,088, 5,482,856, 5,472,693, 5,354,847, 5,292,867, 5,231,026, 5,204,244, 5,202,238, 5,169.939, 5,081,235, 5,075,431, and 4,975,369). For references regarding human antibodies, *see*, *e.g.*, Larrick *et al.*, U.S. Pat. No 5,001,065, for review).

20    Human antibodies of the present invention are may *e.g.* be produced initially in trioma cells. The general approach for producing human antibodies by trioma technology has been described by Ostberg *et al.* (1983) *Hybridoma* 2: 361-367, Ostberg, U.S. Pat. No. 4,634,664, and Engelman *et al.* U.S. Pat. No. 4,634,666.

25    As someone skilled in this field easily realises, the receptor carbohydrates, the ligand polypeptide and the antibodies according to the invention are also useful for a wide variety of diagnostic purposes, such as in immuno assays. Methods, other reagents, amounts *etc* are easily determined by someone skilled in the area on the basis of the information given in this application (for immuno assays, see *e.g.* Harlow and Lane

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(1988) *Antibodies, A Laboratory Manual*, Cold Spring Harbour Publications, New York).

## Detailed description of the drawings

5    In addition to the description given in this section, the drawings will also be commented under "Results" below.

## Figure 1. Identification of rosetting PfEMP1.

Figure 1A rosetting, single *P. falciparum*-infected erythrocyte is seen by light

10   microscopy held by a 5 μm micro-pipette (A, 1). The uninfected erythrocytes are stripped of the infected cell and careful examination confirms that it indeed is infected by a single parasite (A, 2-3).

Figure 1B shows the amplification of a 434-bp band in 4 (from reaction 3, 4, 5 and 7) out of 8 single-infected, rosetting erythrocytes using degenerate primers generated

15   from the primary sequence of the DBL-1 domain of PfEMP1.

Figure 1C shows the amplification pattern with the same primers as in **B** of bulk cultures of rosetting ($R^+$) FCR3S1.2 cultures and the $R^-$ FCR3s/a parasites. Note that the 434-bp product is only seen with the $R^+$ parasites.

Figure 1D shows the hybridisation pattern in Northern blotting of the 434-bp sequence

20   to mRNA extracted from the highly rosetting parasite FCR3S1.2 (84% $R^+$) and the weak hybridisation to the $R^-$ FCR3S/a parasite (9% $R^+$).

Figure 1E shows the autoradiogarph of a Triton-X 100 insoluble, SDS-soluble extract of FCR3S1.2 infected erythrocytes after radio-iodination labelling. PfEMP1 (arrowed) is labelled on FCR3S1.2 infected erythrocytes and is cleaved by low concentrations of

25   trypsin.

## Figure 2. Map of cDNA structure, sequencing clones, deduced amino acid sequence and the location of GAG binding motifs in the rosetting PfEMP1 of FCR3S1.2. A shows the location of 434-bp fragment and the three fragments (I, II and III) which

30   were intially cloned for sequencing. Restriction enzyme-digestion sites are indicated by

arrows. Additional overlapping clones used for sequencing are shown below. B shows the primary structure of the rosetting FCR3S1.2-PfEMP1. It has two Duffy binding-like (DBL) domains (DBL-1 and 4), one cysteine-rich interdomain region (CIDR), one transmembrane (TM) region and one acidic C-terminal segment (ATS). C shows
5    the distribution of amino acids in different regions of FCR3S1.2-PfEMP1.

Figure 3. Rosetting FCR3S1.2-PfEMP1 binds to heparan sulfate. All the gels are 10% SDS-PAGE stained with Coomassie. A shows the expressed GST, DBL-1-GST or ATS-GST after purification on glutathione-sepharose and SDS-PAGE. B shows the
10   binding capacity of different fusion proteins to heparin-sepharose after SDS-PAGE. C shows the inhibition produced by different glycosaminoglycans on the binding of DBL-1-GST to heparin-sepharose followed by SDS-PAGE. D & E show the binding of DBL-1-GST (D) and ATS-GST (E) to monolayers of normal RBC as visualised by a mAb to GST labelled with biotin and FITC-avidin.
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Figure 4. Disruption of pre-formed, natural rosettes with either DBL-1-GST or ATS-GST.
Results are means and standard errors of three experiments.

20   Figure 5. Effect of glycosaminoglycans (GAGs) on P. *falciparum* rosetting. A shows disruption of rosettes exerted by different GAGs. FCR3S1.2 cultures were incubated with GAGs for 1 h at 37° C and compared to control culture. Results are the means and standard error of three separate experiments. B shows the effect of enzyme treatment of uninfected, C-FDA labelled, erythrocytes in a competitive assay of
25   rosette-reformation in the presence of normal erythrocytes and FCR3S1.2 infected pRBC. Results are the means and standard error of three separate experiments; two experiments for neuraminidase.


EXPERIMENTAL

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The following examples are only intended to illustrate the present invention and do not limit the scope of the invention as defined by the appended claims. Throughout the present application, the following abbreviations are used:

PfEMP 1, *Plasmodium falciparum* erythrocyte membrane protein 1; DBL, Duffy
5   binding-like; GST, glutathione S-transferase; GAG, glycosaminoglycan; pRBC, parasitised RBC; ICAM-1, intercellular adhesion molecule 1 ; TM, transmembrane.

## EXAMPLE 1

10   ## Materials and Methods

### The Parasites

The *P. falciparum* parasites were cultured according to standard methods with 10% $AB^+ Rh^+$ serum added to the buffered medium (RPMI supplemented with hepes, gentamycin and sodium bicarbonate). The FCR3S1.2 was cloned with micro-
15   manipulation from the previously limiting-dilution cloned parasite FCR3 (Udomsangpetch, R., B. Wåhlin, J. Carlson, K. Berzins, M. Torii, M. Aikawa, P. Perlmann, and M. Wahlgren. 1989. *Plasmodium falciparum*-infected erythrocytes form spontaneous erythrocyte rosettes. *J. Exp. Med.* 169, 1835-1840). Its rosetting rate was routinely >80% ($R^+$). FCR3S/a was negatively enriched for low rosetting from FCR3
20   using Ficoll-isopaque (<10%, $R^-$). It should be noted that FCR3S was previously called Palo Alto (Uganda) in our publications (Carlson, J., H.P. Ekre, H. Helmby, J. Gysin, B.M. Greenwood, and M. Wahlgren. 1992. Disruption of *Plasmodium falciparum* erythrocyte rosettes by standard heparin and heparin devoid of anticoagulant activity. *Am. J. Trop. Med. Hyg.* 46, 595-602, Udomsangpetch, R., B. Wåhlin, J. Carlson, K.
25   Berzins, M. Torii, M. Aikawa, P. Perlmann, and M. Wahlgren. 1989. *Plasmodium falciparum*-infected erythrocytes form spontaneous erythrocyte rosettes. *J. Exp. Med.* 169, 1835-1840, Scholander, C., C.J. Treutiger, K. Hultenby, and M. Wahlgren. 1996. Novel fibrillar structure confers adhesive property to malaria-infected erythrocytes. *Nature Med.* 2, 204-208, Carlson, J. and M. Wahlgren, 1992. *Plasmodium falciparum*
30   erythrocytes rosetting is mediated by promiscuous lectin-like interactions. *J. Exp. Med.*

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176, 1311-1317, Carlson, J., G. Holmquist, D.W. Taylor, P. Perlmann, and M.

Wahlgren. 1990. Antibodies to a histidine-rich protein (PfHRP1) disrupt spontaneously

formed *Plasmodium falciparum* erythrocyte rosettes. *Proc. Natl. Acad. Sci. U.S.A* 87,

2511-2515, Helmby, H., L. Cavelier, U. Pettersson, and M. Wahlgren. 1993. Rosetting

5    *Plasmodium falciparum*-infected erythrocytes express unique antigens on their surface.

*Infect. Immun.* 61, 284-288). Molecular studies of the "Palo Alto" parasites have

revealed, however, that they are identical to parasites of the FCR3 lineage (Fandeur,

T., S., O. Bonnefoy, Mercereau-Puijalon. 1991. In vivo and in vitro derived Palo Alto

lines of *Plasmodium falciparum* are genetically unrelated. *Mol. Biochem. Parasitol.* 47,

10    167-178).


## Optimisation of single *P. falciparum* RT-PCR

~~Two degenerate primers (DBL-1.1, 5'-GG(A/T)GC(A/T)TG(TC)GC(A/T)CC(A/T)~~
T(A/T)(T/C) (A/C)G -3'; DBL-1.2, 5'-A(A/G)(A/G)T A(T/C)TG (T/A)GG (A/T)AC

15    (A/G)TA (A/G)TC -3' which mapped to the conserved region of all PfEMP1 DBL-1

were modified from the sequences of Su et al. The amplification parameters were first

optimised so that the amplified products were visible with normal ethidium bromide

(EB) staining (Cobb, B. D. and J.M. Clarkson, 1994. A simple procedure for

optimising the polymerase chain reaction (PCR) using modified Taguchi methods.

20    *Nucl. Acid. Res.* 22, 3801-3805). Briefly, one to five parasites, obtained by limiting

dilution, were directly emerged in the RT-PCR buffer (Stratagene) with different

concentration of primers, $MgCl_2$, KCl and Tris-Cl. Both DNA and RNA were released

from the parasites by heating at 93°C for 3 min. The DNA was degraded by addition of

10 U DNase (Stratagene). Reverse transcription was carried out immediately after

25    addition of random primers and reverse transcriptase (Perkin-Elmer). The PCR

reaction was subsequently performed in the same tube. Through comparison of the

amplification efficiency from different reactions, the optimised parameters for single

cell RT-PCR were found to be as follows: 100mM Tris-Cl, pH 8.3, 35mM $MgCl_2$,

500mM KCl, and the final concentration of primers was 1 µM. In the subsequent

30    experiments, individual trophozoite-infect d rosetting erythrocytes were isolated with

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a 5 μm glass-pipette using an inverted microscope. The selected pRBC was stripped of
uninfected RBC and repeatedly grabbed, ejected and turned to conclusively ensure that
it had pigment and that the selected cell was a single trophozoite-infected RBC (see
Fig. 1A). Fifty cycles of amplification at 93°C for 20 seconds, 55°C for 30 seconds and

5    72°C for 1 min were needed for product detection. Several controls were included in
each experiment; one blank control (without parasite(s)) and one without reverse-
transcriptase to rule out the possibility of contamination and amplification due to the
presence of genomic DNA.

10   **RT-PCR with total RNA from bulk-cultured FCR3S1.2 or FCR3S/a and Northern-blot**
**analysis**

Total RNA purification from both FCR3S1.2 ($R^+$) and FCR3S/a ($R^-$) parasite and RT-
PCR was performed as described (Sambrook, J., E.F., Fritsch, and T., Maniatis. 1989.
Molecular Cloning, A laboratory manual, second edition. Cold Spring Harbor

15   Laboratory Press). All the amplified products were cloned with Original TA Cloning
Kit (Invitrogen) and sequenced. Northern blot analysis was carried out using standard
methods (Sambrook, J., E.F., Fritsch, and T., Maniatis. 1989. Molecular Cloning, A
laboratory manual, second edition. Cold Spring Harbor Laboratory Press). Membranes
were probed overnight at 60°C using the 434-bp fragment labelled with a-$^{32}$p-dCTP.

20   Washing was performed under stringent conditions (60°C, 0.1xSSC) and the blots
were examined in a Molecular Dynamics phosphoimager.

**Cloning and Sequencing of the whole cDNA**

A specific upstream primer (L-6, 5'-GAC ATG CAG CAA GGA GCT TGA TAA -3')
in the 434-bp sequence and a downstream primer (L-5, 5'-CCA TCT CTT CAT ATT
CAC TTT CTG A -3') mapping to the conserved sequence of ATS were generated and
reverse transcription was carried out as described above. PCR was performed with the
Expand™ High Fidelity PCR System (Boehringer Mannheim). A single 4.9-kb
fragment was amplified, which was digested into three fragments with Hind III and

30   EcoR V and cloned into the pZErO-1 vector (Zero Background, Invitrogen). The

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sequencing was performed with LongRanger™ gel (FMC) on an A.L.F. Sequencer
(Pharmacia). The 5' region of the FCR3S1.2-*var*1 transcript was cloned by screening a
cDNA library (Schlichtherle, unpublished) with the 434-bp fragment as probe and
seven overlapping fragments were sequenced. The 3' terminal region was cloned by

5     nested RT-PCR. Reverse transcription was primed with oligo-dT and PCR was
performed with a specific 5' primer (P-1, 5'-CTT TCG ACT CTA CCA TCC T -3') 
upstream of TM region and a 3' primer (P-4, 5'-TTA GAT ATT CCA TAT ATC TGA 
TA -3') mapping to the C-terminal sequence of FCR3 (*var* 2) PfEMP 1. Five 
overlapping fragments were sequenced. Fourteen overlapping clones were in total 

10    sequenced in both directions in order to ensure that the sequence was correct and was 
transcribed from a single gene.


## Sequence analysis

The DNA and amino acid sequence analysis (editing, translation, peptidesort, plot,
15    etc.) was performed with the Genetic Computer Group (GCG) (Devereux, J., P.
Haeberli, and O. Smithies. 1984 A comprehensive set of sequence analysis programs 
for VAX. *Nucl. Acid. Res.* 12, 387-395) program. Alignment of the deduced amino 
acid sequence with the published PfEMP1 sequences in the GenBank was made to 
identify the sequence.  The identification of potential GAG-binding motifs in 

20    FCR3S1.2-*var*1 and in the other published PfEMP1 sequences was done by firstly 
searching for the presence's of either of the 4 amino-acid motifs KK, KR, RK, or RR, 
and secondly  manually checking each of the identified sequences for the presence of 
the motifs XBBXBX or XBBBXXBX where B is a basic amino acid (K,R,H) and X is 
a hydropathic residue. A certain degree of liberty concerning the location of the basic 

25    residues in the motifs is acceptable (Cardin, A. D. and H.J.R. Weintraub, 1989. 
Molecular modeling of protein-glycosaminoglycan interactions. *Arteriosclerosis* 9, 21-
32).


## Expression of DBL-1 or ATS using the pGEX-4T-1 vector

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Both DBL-1 and ATS fragments were amplified by specific primers (Ex-1.1, 5'-ATC
GAA TTC TGC AAA AAA GAT GGA AAA GGA A -3' and D-1, 5'-GTA TTT TTT
TTG TTT GTC AAA TTG -3' for DBL-1; Ex-2, 5'-ATC GAA TTC TCT GAA AAT
TTA TTC CAA A -3' and P-4 for ATS). The amplified fragments were inserted into

5     the EcoR I cloning site of pGEX-4T-1 downstream of the glutathione S-transferase
sequence. The *E.coli* BL21 was used as the expression strain. Expression of both
fusion proteins was induced with 0.1mM IPTG at 30°C for 4h and the fusion proteins
were purified on glutathione sepharose (Pharmacia) as described in the instructions
provided by the manufacturer (GST Gene Fusion System, Pharmacia). The expression

10    constructs were sequenced by cycle sequencing to check that the recombinant
plasmids were of the expected sequences in the correct reading frames. Thrombin
cleavage of the fusion proteins was performed according to a standard procedure.
Western-blot analysis of DBL-1-GST and ATS-GST fusion proteins was with a biotin
labelled anti-GST mAb (clone GST-2, IgG2b, Sigma) and ALP-avidin (Sigma) to

15    reveal the pattern of protein expression. Although the induction of expression was at a
low temperature and the purification was in the presence of a cocktail of enzyme
inhibitors (0.5mM EDTA, 1mM Pefabloc®SC (AEBSF), Boehringer Mannheim),
there was still some breakdown of the DBL-1-GST. The fusion proteins, stained by the
anti-GST mAb, decreased with thrombin treatment. This information together with the

20    knowledge that the plasmids were of the expected sequences ensured that the fusion
proteins indeed were the correct ones.


Heparin binding and blocking assay

The potential binding of the fusion proteins to heparin was studied by mixing either

25    DBL-1-GST, ATS-GST fusion protein or GST alone (20 µl, 150 µg/ml in PBS) with
20 µl of 50% heparin-sepharose (Pharmacia) and incubating for 5 min at room
temperature in the absence of serum; binding to un-coupled sepharose was used as
control. The heparin-sepharose and protein mixture was washed 3 times in large
volumes of PBST buffer (PBS plus 0.05% Tween-20) before extracting bound proteins

30    in loading-buffer for 10% SDS-PAGE. Inhibition of DBL-1-GST fusion protein

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binding to heparin with heparin, heparan sulfate or chondroitin sulfate (Lövens Kemiske Fabrik, Denmark, more than 90% pure) was also tested. Twenty µl of 150 µg/ml DBL-1-GST fusion protein was mixed separately for 5 minutes with an equal volume of heparin, heparan sulfate, or chondroitin sulfate, titrated from 10 to 0.5

5    mg/ml, before the addition of heparin sepharose. The inhibitory activities were checked by SDS-PAGE.


## Erythrocyte binding and blocking assay

Ten-well immunofluorescence glass-slides were precoated with 10% poly-L-lysine in

10   PBS for 30 min. Monolayers of RBC were made by addition of 20 µl of 0.5% 3x washed bloodgroup O Rh$^+$RBC in PBS to each well. Twenty µl DBL-1-GST , ATS-GST or GST alone (80 µg/ml) in PBS was added to the wells for 30 min. The DBL-1-GST fusion protein was in subsequent experiments incubated in the presence of heparin, heparan sulfate or chondroitin sulfate (titrated from 20 to 8 mg/ml) to study

15   the inhibitory activity of each GAG. Slides were washed 3 times with PBS and the fusion protein-binding was detected with the biotin-labelled anti-GST mAb and an ExtraAvidin$^®$FITC conjugate (Sigma). The fluorescence was assessed in a Nikon Optiphot-2 UV microscope, using a x10 ocular and an oil lens with a magnification of x100.

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## Rosette disruption assay

The assay was performed essentially as described (Carlson, J. and M. Wahlgren, 1992. *Plasmodium falciparum* erythrocytes rosetting is mediated by promiscuous lectin-like interactions. *J. Exp. Med.* 176, 1311-1317). The recombinant fusion proteins (25 ml in

25   PBS of DBL-1-GST or ATS-GST) were added to 25 µl aliquots of a ≈80% rosetting culture of FCR3S1.2 in a micro-titre plate. The mixtures were incubated for 30 min. at 37°C after which the rosetting rate was scored and compared to mock treated controls after staining with acridine orange.


30   ## Enzyme treatment of normal RBC

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Human bloodgroup O Rh$^+$ erythrocytes ( 5% suspension in RPMI) were, previous to C-FDA labelling (Carlson, J., G. Holmquist, D.W. Taylor, P. Perlmann, and M. Wahlgren. 1990. Antibodies to a histidine-rich protein (PfHRP1) disrupt spontaneously formed *Plasmodium falciparum* erythrocyte rosettes. *Proc. Natl. Acad. Sci. U.S.A* 87,

5      2511-2515), incubated for 60-90 min with either heparinase III (25° C, pH 7.5; Sigma), chondroitinase ABC (37° C, pH 8.0; Sigma) or with C. *perfringens* neuraminidase (37° C, pH 6.0; Sigma). Cells were washed three times after treatment and resuspended in complete malaria medium, with 10% serum. Control erythrocyte suspensions were mock treated, washed and incubated as above.

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## Results

## Identification of a novel rosetting PfEMP1-variant

With the aim of identifying and isolating a PfEMP1-variant of a rosetting parasite, a single-cell RT-PCR assay was developed using micro-manipulation. Since the PfEMP1

15      messages are mostly expressed at ring stage, it is a difficult task to amplify cDNA from single trophozoites. However, the present inventor was successful in four out of eight pRBC studied and, when amplified, it was always a 434-bp sequence that was found (Fig. 1B). This was confirmed by amplification of *var* transcripts from bulk cultures, where the 434-bp amplificate was again detected unique to the rosetting *P.*

20      *falciparum* and was not found present in non-rosetting parasites (Fig. 1C). Both the single cell- and the RT-PCR with the total RNA from the bulk cultures were repeated several times to make sure that the results were reproducible. Further, the 434-bp product was also found present in 7 out of 12 ring stage-parasites using single-cell RT-PCR (data not shown). Sequence analysis revealed that the amplified product encoded

25      the semi-conserved sequence of the 5′ located DBL-I domain of the *var* genes. Ten 434-bp sequences obtained from separate amplifications were found to be identical. 9 distinct PCR-amplified *var* transcripts from non-rosetting parasites were also isolated subcloned and sequenced. They were in all instances different from the 434-bp sequence (not shown). Northern-blot analysis with mRNA from the FCR3S1.2 clone

30      and the 434-bp sequence as the labelled probe revealed a transcript of about ≈7.5-kb,

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the difference with the size of the coding cDNA of 6.7 kb being accounted for by a

relatively larg untranslated 5' region (Sundström et al, unpublished). The weak

hybridisation seen with mRNA from R-parasites is probably due to hybridyzation to a

different transcript (Fig. 1D). Thus, according to the present invention, a unique

5   PfEMP1 transcript has been found in rosetting parasites.


## cDNA structure of rosetting PfEMP1

The entire coding region of the *var*- transcript containing the 434-bp motif was

assembled with the 13 overlapping fragments and the coding sequence was found to

10  be composed of 6684-bp (SEQ ID NO:1). 6684-bp encodes a 2228 aa polypeptide

with an estimated molecular weight of 260 kDa. A single trypsin-sensitive polypeptide

of a similar size was seen after $^{125}$I- lactoperoxidase surface-labelling of the FCR3S1.2

(Fig.1E).


15  In an in-depth analysis of the expressed FCR3S1.2-*var*1 transcript, it was found that

the overall structure was similar to the published *var*-sequences. However, the

sequence of the novel variant according to the present invention differs substantially

from the prior sequences in that it is shorter than most previous sequences, as it

contains two DBL-domains (DBL-1 and 4), rather than four, separated by a cysteine-

20  rich interdomain region (CIDR) (Fig. 2B). Both the TM region and the negatively

charged, acidic C-terminal segment (ATS) of the FCR3S1.2-*var*1 exhibited a certain

degree of homology (≈80%) to previously published sequences. However, neither the

potentially variable sequences of 434-bp *var*-gene fragment nor the other regions of the

cDNA were contained in other published *var*-sequences, such as the one described in

25  WO 96/33736.


## Rosetting PfEMP1 contains clusters of glycosaminoglycan-binding motifs

The FCR3S1.2-*var*1 sequence was examined for the presence of potential GAG-

binding motifs. 19 potential GAG-binding sequences were identified and it was found

30  that ≈95% of them (18/19) were located in the N-terminus. 8/19 were situated in DBL-

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1, 5/19 in the CIDR C-terminally to DBL-1 and 5/19 in DBL-4. Only one potential GAG-binding motif was seen beyond the TM region (SEQ ID NO:1). The N-terminal distribution of these aa clusters is therefore consistent with the accessibility of the FCR3S1.2-var1 at the surface of the pRBC.Thus, this may be the molecular

5    background to rosetting and thereby explain why rosettes are sensitive to heparin.


## Rosetting PfEMP1 binds to a heparan sulfate-like GAG

To confirm the above findings experimentally, two domains of the FCR3S1.2-var1 transcript were subsequently expressed: one which had 8 GAG-binding motifs (DBL-

10    1, 1008-bp corresponding to 336 aa) and one which was the highly charged, acidic C-terminal sequence (ATS) that lacks GAG-binding motifs (1353-bp corresponding to 451 aa). The purified DBL-1-GST efficiently bound to heparin-coupled sepharose already after a few minutes at room temperature while the ATS-GST fusion protein did not (Fig. 3B) and a second DBL-1-GST construct covering a distinct var sequence (var

15    2, Su, X.-Z, V.M. Heatwole, S.P. Wertheimer, F. Guinet, J.A. Herrfeldt, D.S. Peterson, J.A. Ravetch, and T.E. Wellems, 1995. The large diverse gene family var encodes proteins involved in cytoadherence and antigenic variation of Plasmodium falciparum-infected erythrocytes. Cell 82, 89-100), which lacks GAG-binding motifs, also failed to bind to the heparin-matrix (not shown). The adhesion could dose-dependently be

20    competed out with heparin or heparan sulfate but not with chondroitin sulfate, another negatively charged erythrocyte surface expressed GAG (Fig. 3C ). Taken together, this indicates that the binding of DBL-1 to heparan sulfate is dependent on structure and not merely ionic interactions between the two molecules. Heparan sulfate or a heparan sulfate-like GAG therefore seems to be the binding target for the novel PfEMP1-

25    variant.


## Rosetting PfEMP1 binds directly to erythrocytes

To study the potential binding of the recombinant PfEMP1 to erythrocytes we formed monolayers of uninfected erythrocytes on glass-slides. The cells were subsequently

30    incubated with different concentrations of the fusion proteins. While the ATS-GST did

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not bind to the erythrocytes as detected with an anti-GST mAb, the DBL-1-GST gave a distinct surface staining of all the uninfected erythrocytes (Fig. 3D, E). This was titratable and could be inhibited with small amounts of heparin or heparan sulfate but not with the related GAG, chondroitin sulfate which suggests that the binding was

5        specific. Heparan sulfate has previously been suggested to be present on human erythrocytes (Trybala, E., B. Svennerholm, T. Bergström, S. Olofsson, S. Jaensson, and J.L. Goodman. 1993. Herpes simplex virus type 1-induced hemagglutination: glycoprotein C mediates virus binding to erythrocyte surface heparan sulfate. J. Virol., 67, 1278-1285, Baggio,B., G. Marzaro, G. Gambaro, F. Marchini, H.E. Williams, and

10       A. Borsatti. 1990. Glycosaminoglycan content, oxalate self-exchange and protein phosphorylation in erythrocytes of patients with idiopathic' calcium oxalate nephrolithiasis. Clinical Science, 79, 113-116) and we therefore conclude that the DBL-1-GST fusion protein binds with heparan sulfate specificity to both to the solid-phase matrix as well as to normal erythrocytes.

15

Recombinant DBL-1 disrupts rosettes and blocks rosette-reformation

The effect of the DBL-1 fusion protein on pre-formed rosettes was studied in order to confirm the biological role of PfEMP 1 in rosetting. Aliquots of a highly rosetting FCR3S1.2 culture was incubated with decreasing concentrations of DBL-1-GST or

20       ATS-GST. DBL-1 caused a dose-dependent rosette reversion with 40-50% reversion at ≈50 μg/ml while the ATS-GST did not show any effect (Figure 4). The rosettes were not reformed upon prolonged incubation.

Rosetting is dependent on heparan sulfate

25       To establish the role of heparan sulfate also in rosetting we studied the disruptive activity of different GAGs on FCR3S1.2 rosettes. FCR3S1.2 rosettes were sensitive to both  heparin and to heparan sulfate, but neither chondroitin sulfate A, keratan sulfate, nor hyaluronic acid had any effect on the rosettes (Fig. 5A). Chondroitin sulfate B had a slight effect only at high concentrations (Fig. 5A). These findings were confirmed by

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enzyme treatment of the uninfected erythrocyte: heparinase, but not chondroitinase or neuraminidase, treatment blocked the binding (Fig. 5B).


## EXAMPLE 2

5    Materials and methods

*Parasites and rosetting* - Two distinct highly rosetting *P. Falciparum* laboratory strains were used: FCR3S1 (previously named R$^+$PA1) and TM 284 (isolated in Thailand, 1990). Parasites were cultivated according to standard procedures (Udomsangpetch *et al.* 1989). Field isolates were obtained from patients attending the

10    Albert Schweitzer Hospital, Lambaréné, Gabon between April and June 1996. Patients with a *P. falciparum* asexual stage parasite count superior to 10.000/µl blood were included in the study. A venous or capillary blood sample was taken after patients or parents informed consent. Blood was washed three times with RPMI and cultivated according to standard procedures (Udomsangpetch *et al.* 1989). Assessment for

15    parasite growth and rosetting was made every 6-8 hours using a Leitz UV-light microscope. Briefly, an aliquot of parasite culture was mixed with a small amount of acridine orange and the sample was analyzed. Assays were performed on rosetting isolates when the majority of parasites in culture had reached the trophozoite stage. The rosetting rate was expressed as the number of infected erythrocytes in rosettes

20    relative to the total number of late stage infected erythrocytes. Three hundred infected cells were counted. Parasitemia was calculated as the number of infected erythrocytes relative to the number of uninfected erythrocytes.


*Saccharides* - A number of GAGs, polysaccharides, heparin mono- and disaccharides

25    were assessed for rosette disrupting activity. Heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate (all from porcine intestine) as well as fucoidan (*Fucus vesiculosus*), dextran sulfate (Mol. Wt. 8.000), keratan sulfate (bovine cornea), hyaluronic acid (rooster comb), heparin/HS mono- and disaccarides (produced by digestion of heparin and HS by various heparinases) were purchased from Sigma (St.

30    Louis, USA). Dextran (Mol. Wt. 60.000) was from Medisan Pharmaceuticals,

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Stockholm, Sweden. High molecular weight heparin with low affinity for antithrombin III (HMW-LA heparin) was produced by gel filtration of standard heparin on Sephacryl S-200 (Pharmacia, Uppsala, Sweden) followed by passage over an antithrombin-Sepharose 4B column (Carlson *et al.* 1992). Modification of bovine lung

5    heparin and structural characterizations were performed as described (Feyzi *et al.* 1997).

*Treatment of erythrocytes and infected erythrocytes* - Treatments of blood group O Rh[+] red blood cells (RBC) and cultures were performed with heparinase III (0.033 IU/ml,

10    25° C, pH 7,5), chondroitinase ABC (20 IU/ml, 37° C, pH 7,5), *Clostridium perfringens* neuraminidase (0.1 IU/ml, 37 ° C, pH 6,0) and phospholipase C (0.1 IU/ml, 30 ° C, pH 7,5). The enzymes were purchased from Sigma and Seikagaku Corporation. Rosetting rate was assessed after 210 minutes incubation with enzyme, without changing any cinditions in the culture and was compared to mock treated

15    control cultures. No extracellular parasites or RBC gross morphology change was observed at concentrations and incubation time used. Digestion with trypsin (100 IU/ml, 37° C, pH 7,5) and chymotrypsin (100 IU/ml, 37° C, pH 7,5) was stopped with soybean protease inhibitor (Sigma) after 20 minutes incubation and rosetting rate was assessed. Sodium periodate in RPMI was added to cultures and uninfected RBC at 10

20    mM concentration. Rosetting rate was checked after 15 minutes incubation at room temperature and compared to mock treated control cultures.

*Rosette disruption assays* - Assays on wild isolated were performed on cultures presenting a rosetting rate superior to 20% (range 23.6-86.2%, mean 48.6&) and

25    parasitemia 1-10%. For laboratory strains, parasite cultures with rosetting rates of 50-70% and parasitemia 5-8% were used Aliquots (12.5-25 μl) of parasite cultures were mixed with equal amounts of sulfated glycoconjugate compound in RPMI in a 96-well microtiter plate, at final concentrations indicated. After incubation for 30 minutes at 37° C, assessment for rosetting was made as described above. The rosetting rate was

30    compared to that of culture mixed with RPMI.

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*Competition assay using carboxy-fluorescein diacetate (C-FDA) labelled erytrhocytes*
- In order to measure the relative capacity of treated RBC to form rosettes, a
competition assay was performed as described by Carlson and Wahlgren (Carlson *et al.*
1992), with some modifications. Blood group O Rh$^+$ RBC were pre-treated for 60-90

5    min by enzymes or periodate as described above and mixed with a small amount of C-
FDA (Sigma) stock solution to a final concentration of 250 µg/ml. The cells were
incubated for 10-15 minutes at room temperature, then washed three times with RPMI
and finally resuspended at 5% hematocrit in malaria culture medium containing 10%

10   AB$^+$ serum. Controls were mock treated and labelled as described above.

Fresh rosetting cultures grown in blood group O Rh$^+$ RBC from the same donor were
resuspended as above. A 25 µl aliquot of culture was mixed with an equal volume of
the C-FDA-labelled uninfected RBC that had been subjected to enzyme or periodate

15   pre-treatment. The rosettes were disrupted mechanically by drawing the cells through
an injection needle (diameter 0.60 mm) 6-8 times. After 30 minutes incubation rosette
reformation was assessed and the proportion of labelled to unlabelled RBC in rosettes
claculated. Assays were considered valid when the ratio of labelled/unlabelled RBC in
the assay was 0.9-1.1 and the ratio of labelled/unlabelled RBC participating in rosettes

20   was 0.9-1.1 for control samples.

## RESULTS
*Disruption of rosettes from laboratory strains FCR3S1 and TM284 by SGs* - Rosette
disruption by GAGs and three other related polysaccharides (dextran, dextran sulfate

25   and fucoidan) performed on two laboratory strains revealed differences in GAG-
sensitivity (Table I). Although having a similar molecular structure, some compounds
totally lacked rosett  disrupting activity at high concentrations (1-10 mg/ml), while
other compounds were potent rosette disrupters even at lower concentrations (1-10
µg/ml; Table I and not shown). FCR3S1 exhibited high sensitivity for heparin and HS,

30   whereas dermatan/chondroitin sulfate had little or no effect. On the other hand, TM

284 exhibited relatively higher sensitivity for dermatan/chondroitin sulfate than FCR3S1 and was less sensitive to HS/heparin (Tabl I). Still, heparin disrupten 80% of TM 284 rosettes at 1 mg/ml (not shown).

5    *Sensitivity of wild isolates to SGs* - Thirtytwo patient isolates were characterized in order to analyze the properties of fresh *P. Faciparum* parasites. The sensitivity of rosettes towards the different GAGs and other sulfated polysaccharides was clearly isolate dependent. In general, two major groups could be discerned (Fig. 1). One group was more sensitive to the glucosaminoglycans heparin and heparan sulfate, and

10   somewhat less to the galactosaminoglycans (chondroitin sulfate and dermatan sulfate). For the second group of isolates the galactosaminoglycans were more effective competitors than the glucosaminoglycans. For both groups, fucoidan was a strong rosette disrupter in contrast to dextran sulfate which was much less effective that GAGs in most of the strains. This picture indicated that charge was an important but

15   not the only determinant in rosette disruption. One the whole collection of wild isolates , fucoidan was the most effective rosette disrupter for most isolates and chondroitin sulfate the least effective, whereas dermatan sulfate, heparin, HS and dextran sulfate had an intermediate position. This was also true at other concentrations tested (10-100 µg/ml; not shown). No clear correlation was observed between differecnes in SG

20   sensitivity of the isolates and parasitemia, rosetting rate or ABO blood group of the patient (not shown).

     *Disruption of rosettes by modified heparin* - In order to investigate the relative importance of different molecular features, modified heparin was tested for rosette

25   disruption on the heparin/HS sensitive FCR3S1 strain. When *N*-dulfate groups were replaced by the charge neutral acetyl group, heparin was ≃ 100 fold less efficient in rosetting disruption compared to the native form (Fig. 2). In contrast to this marked effect, *O*-desulfation seemed to be much less important, as removal of either 2-*O*- or 6-*O*-sulfate groups resulted in an ≃ 10 fold reduction ofd disruption efficiency. Even the

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most completely *O*-desulfated sample with only about 30% of disaccharide units still 2-*O*-sulfated had a similar order of efficiency.

*Sensitivity of rosettes to disaccharides* - As the effect of heparin seemed to be strongly
5    related tpo the presence of the *N*-sulfate group, we tested whether the individual disaccharides would have a rosette reverting capacity. Different disaccharides that are gained by heparinase cleavage of heparin and HS were tested in the rosetting assay. The most acitve ones all contained the *N*-sulfated glucosamine, while the efficiency of the disaccharides was reduced by acetylation of the amino group corroborating data
10    with the modified heparins (Fig. 3). The *O*-sulfation on the other hand had only limited influence. While 6-*O*-sulfation seemed to be less important, the 2-*O*-sulfate had some positive effect in addition to the *N*-sulfate. Additionally, heparin disaccharides II-H ($\Delta$HexA-GlcN(6-OSO$_3$)), III-H ($\Delta$HexA(2-OSO$_3$-GlcN), and IV-H ($\Delta$HexA-GlcN), all lacking *N*-sulfation and with a positively charged amino group, exhibited no rosette
15    disrupting effect (not shown).

One active and one inactive disaccharide were studied in detail: complete rosette reversion was obtained by the *N*-sulfated disaccharide IV-S ($\Delta$HexA-GlcNSO$_3$ at 45 mM while the *N*-acetylated disaccharide IV-A ($\Delta$HexA-GlcNAc) completely lacked
20    effect (Fig. 4A).

In a preliminary experiment the most predominant heparin disaccharide recovered after heparinase digestion, I-S ($\Delta$HexA(2-OSO3)-GlcNSO$_3$(6-OSO$_3$)), was titrated against heparin for rosette disruption on strain FCR3S1. At a concentration of 30 mM
25    disaccharide, the rosettes were disrupted to the same extent as with heparin at 1 mg/ml (corresponds to $\sim$ 70 µM heparin molecules), e.e. the heparin chain was around 20 fold more efficient on a weight basisk and around 500 fold more efficient on a molar basis. When wild type isolates were tested with this concentration of disaccharide, in comparison with the other competitors, it exhibited a similar pattern as heparin (Fig. 1).

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*Sensitivity of rosettes to monosaccharides* - When glucosamine was tested, the *N*-sulfated monosaccharides gave ~50% rosette reversion at 50 mM and additional sulfate groups and increasing negative charge did not potentiate rosette disruption. Some activity could also be attributed to *N*-acetylated glucosamine with an *O*-sulfate ester at

5    C-6 (Fig. 5B). Similar results were obtained when the TM 284 strain was tested, though slightly higher concentrations were required (not shown). Also, *N*-sulfated galactosamine (not present in any GAG) lacked rosette disrupting activity, whereas *N*-sulfated glucosamine (common in heparin/ HS) caused total rosette disruption (Fig. 4B).

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*Glycan oxidation and enzymatic treatment of whole cultures* - Different treatments of infected erythrocyte cultures caused changes in rosetting behavious withour any gross change on RBC morphology on both strains tested (Fig. 6A). When the cultures were treated with the proteases trypsin or chymotrypsin rosetting was abolished completely,

15    indicating that either receptor or ligand in the adhesion complex is a highly protease-sensitive protein. Heparinase III, which selectively cleaves -4GlcNRα-[1→4]-GlcA1-(where R can be either N-acetamido or N-sulfo) linkages of heparan sulfate chains (Linhardt *et al.* 1990) resulted in a drastic reduction of the rosetting capacity of FCR3S1 and a somewhat lower decrease of TM 284 rosetting. Chondroitinase ABC,

20    cleaving GalNAcα-[1→4]-GlcA bonds in chondroitin sulfate had only effect on the rosetting of the TM284 strain. No effect was seen by either neuraminidase or phospholipase C treatment. Sodium periodate which would oxidize any glycan type with vicinal hydroxyl groups had an intermediate effect on both parasite strains.

25    *Glycan oxidation and enzymatic treatment of uninfected RBC* - In order to assess whether above results can be attributed to the infected RBC *per se* or to the uninfected RBC that participate in rosetting, competition assays were achieved wioth pre-treated, C-FDA labelled uninfected RBC. Sodium peri date treatment of uninfected RBC decreased their rosette binding capacity 4 tim s compared to non-treated RBC. The

30    rosette binding capacity of heparinase III treated RBC was also decreased 4-5 fold.

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Treatment with heparinase III in presence of soybean protease inhibitor did not reverse

the effect, and addition of HS to heparinase completely blocked the heparinase activity

(not shown). Also, typsin and chymotrypsin treatment affected the binding capacity,

with a 2-5 fold decrease, but less than the treatment of the whole culture. This finding

5    suggestes that the primary protease sensitive molecule is the ligand on the infected

RBC, while not all receptor structures on the uninfected RBC are sensitive to

proteolytic degradation. This can be either due to a reduced protease sensitivity of

glycosylated proteins/proteoglycans or because the receptor glycans would sit on a

non-proteinaceous core. For the TM 284 strain, a two fold decrease of binding

10   capacity was observed after chondroitinase ABC treatment, whereas FCR3S1 was

unaffected (Fig. 6 B).


## DISCUSSION

The present invention discloses the identification of a novel PfEMP1-variant as the

15   rosetting ligand and heparan sulfate, or a heparan sulfate like molecule, as the rosetting

receptor. A single cell RT-PCR technique was developed in order to investigate the

association of expression of the novel PfEMP1-variant with rosetting-binding at the

single cell level. The PfEMP 1 messages were found to be present in ring stage

parasites, in trophozoites and in schizonts, although it was less abundant in the more

20   mature stage parasites. However, these were studied in great detail as they do form

rosettes and rings do not. One PfEMP 1 mRNA species was amplified from single

rosetting parasites and the appearance of the same fragment in amplifications with total

RNA from R$^+$ bulk cultures, but not with total RNA from non- or low rosetting

parasites, indicated that the message was unique to rosetting parasites. It was evident,

25   however, that the parasites were not homogeneous even in cultures of a high rosetting

rate suggesting that the non-rosetting parasites may express different PfEMP 1s.

Similarly, although a strong hybridisation to a ≈7.5 kb message from the rosetting

parasites was found, there was still a small hybridisation to a slightly smaller speci s

with RNA from the R⁻ parasites. This probably reflects the transcription of a different

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*var* gene, since the FCR1S.2 *var*1 sequence was not found in the amplified products
from R- parasites.

5      Cloning of full length cDNA from eukaryotic cells is always difficult. For example,
Harshman *et al.* (1995) *Hum. Mol. Genet.*4: 1259-1266, have shown that different
methods of identifying cDNAs from a genomic region result in a surprisingly different
array of candidate genes. To be able to achieve this, the RT-PCR parameters were first
optimised, so that most PfEMP1 transcripts could be amplified. With these conditions
and a primer sequence from the unique 434-bp rosetting PfEMP1 cDNA, one large
10     single down-stream fragment of 4.9-kb of cDNA was amplified, which upon the
digestion with EcoR I and Hind III was fragmented into distinct bands indicating that
the amplified product was composed of one sequence. The 4.9-kb cDNA species was
subsequently confirmed to be a single amplificate by sequencing. Rosetting-PfEMP1
specific sequences of both the variable region, upstream to the trans-membrane
15     domain, and of the 434-bp fragment were used to obtain the sequences of overlapping
regions of the 3´ and the 5´ regions of this transcript. Five overlapping fragments were
sequenced to ascertain the 3´ overlap and seven to ascertain the 5´ overlap. The
correctness was further checked by RT-PCR with specific primers flanking the
overlapping regions after the assembly of the entire coding sequence. Each of the 14
20     PCR-amplified fragments were sequenced in both directions and the overlapping
fragments were found to be correct and identical in all regions. It is therefore,
hypothesized that the assembled transcript is indeed derived from one single gene.

The high sensitivity of rosettes to heparin and above specified glycosamino glycans
25     (GAGs) made the present inventors speculate that the parasite uses sulfated
carbohydrates as the rosetting receptor. The appearance of potential GAG binding
motifs in this novel PfEMP 1 sequence was therefore checked after the assembly of the
transcript. Clusters of GAG binding motifs, the consensus sequence of which have
previously been proposed by others, were then identified. All the motifs showed an
30     identical or a similar composition as other published heparin or heparan sulfate binding

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motifs found in both malaria parasites and other organisms (Sinnis, P., P. Clavijo, D.
Fenyö, B.T. Chait, C. Cerami, and V. Nussenzweig. 1994. Structural and functional
properties of region II-plus of the malaria circumsporozoite protein. *J. Exp. Med.* 180,
297-306, Jackson, L.R., J.S. Busch, and D.A. Cardin. 1991. Glycosaminaoglycans:

5    molecular properties, protein interactions, and role in physiological processes. Physiol.
Rev., 71, 481-530). The expressed DBL-1, which had 8 motifs, bound to heparine-
sepharose, to the membrane of normal RBC, disrupted naturally formed rosettes and
blocked rosette-reformation while the ATS fusion protein did not. The inhibitory
activity of heparan sulfate as well as the inability of chondroitin sulfate to inhibit or

10   block the attachment informed us that it was the molecular structure of the GAG that is
important for binding. In complementary experiments, the DBL-1 region of a second
var-gene transcript (*var*-2) which lacks GAG-binding motifs was expressed and found
**not** to bind to the heparin-sepharose (Sahlen, not shown), which also support a
functional role of the GAG binding sequences in the rosetting DBL-1.

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The rosette reversion obtained by the addition of recombinant DBL-1 to naturally
formed rosettes indicated that the parasite uses the novel PfEMP1-variant as the main
rosetting ligand. The deletion of rosetting by heparinase treatment of normal RBC
provided further confirmation about this specific ligand-receptor interaction, indeed

20   suggesting that heparan sulfate, or at least a heparan sulfate-like molecule, is involved
in the binding. Heparinase treatment also disrupted the rosettes of other strains of
parasites (TM180, TM284), while the rosettes of the strain R29 were not affected (not
shown), findings which are in concordance with those of Rowe *et al* (Rowe, J.A., J.M.
Moulds, C.I. Newbold, and L.H. Miller. 1997. *P. falciparum* rosetting mediated by a

25   parasite-variant erythrocyte membrane protein and complement-receptor 1. *Nature*
388:292-295). In separate experiments, heparin-derived mono- and di-saccharides
which were of the same net charge but had th ir sulfate groups at different positions
were studied for their rosette-disrupting activities. Those with the sulfate groups in the
same position as heparan sulfate inhibited binding while the others did not (Barragan

30   unpublished and see above). Further, while the N-sulfated mono-saccharide

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glucosamine, a component of heparan sulfate, had a good anti-rosetting activity the identically N-sulfated galactosamine had none (Barragan et al, unpublished). Heparin has also been found to disrupt rosettes from 27 out of 54 fresh *P. falciparum*-isolates (50%) suggesting that heparan sulfate is used by these parasites as rosetting receptors

5     (Carlson, J., H.P. Ekre, H. Helmby, J. Gysin, B.M. Greenwood, and M. Wahlgren. 1992. Disruption of *Plasmodium falciparum* erythrocyte rosettes by standard heparin and heparin devoid of anticoagulant activity. *Am. J. Trop. Med. Hyg.* 46, 595-602). The receptors used by the heparin-resistant fresh isolates maybe other GAGs, as it has been found that rosettes of most parasites may be disrupted by either one or the other of the

10    human GAGs (Barragan, unpublished). Further, other sulphated glycans, such as fucoidan, disrupted rosettes of almost all fresh isolates (Fig. 1). Thus, according to the present invention, it has been found that FCR3S1.2 uses heparan sulfate, or a heparan sulfate like molecule, on the erythrocyte surface as a rosetting receptor and that the other GAGs may be receptors of other parasites.

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Heparan sulfate is a molecule presents on all cells, including erythrocytes (Trybala, E., B. Svennerholm, T. Bergström, S. Olofsson, S. Jaensson, and J.L. Goodman. 1993. Herpes simplex virus type 1-induced hemagglutination: glycoprotein C mediates virus binding to erythrocyte surface heparan sulfate. J. Virol., 67, 1278-1285, Baggio,B., G.

20    Marzaro, G. Gambaro, F. Marchini, H.E. Williams, and A. Borsatti. 1990. Glycosaminoglycan content, oxalate self-exchange and protein phosphorylation in erythrocytes of patients with idiopathic' calcium oxalate nephrolithiasis. Clinical Science, 79, 113-116); $4 \times 10^6$ molecules have been suggested to be expressed at the surface of normal hepatocytes (Kjellen,L., Å. Oldberg, and M. Höök. 1980. Cell

25    surface heparan sulfate. J Biol. Chem. 21, 10407-10413). The density on the RBC should also be high if this indeed is the receptor used by the parasite for rosetting. The involvement of GAGs as receptor structures for other cell-to-cell interactions of *P. falciparum* has previously been suggested e.g. for endothelial binding , for liver- and erythrocyte invasion (Sinnis, P., P. Clavijo, D. Fenyö, B.T. Chait, C. Cerami, and V.

30    Nussenzweig. 1994. Structural and functional properties of region II-plus of the

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malaria circumsporozoite protein. *J. Exp. Med.* 180, 297-306, Kulane, A., H-P. Ekre,
P. Perlmann, L. Rombo, M. Wahlgren, and B. Wahlin. 1992. Effect of different
fractions of heparin on *Plasmodium falciparum* merozoite invasion of red blood cells
*in vitro. Am. J. Trop.Med. Hyg.*, 46, 589-594, Robert, C., B. Pouvelle, P. Meyer, K.

5      Muanza, A. Scerf, and J. Gysin. 1995. Chondroitin-4-sulfate (proteoglycan) as
*Plasmodium falciparum*-infected erythrocyte adherence receptor of brain
microvascular endothelial cells. *Res. Immunol.* 146, 383-393, Rogerson, S.J., S.C.
Chaiyaroj, K. Ng, J.C. Reeder, and G.V. Brown. 1995. Chondritin sulfate A is a cell
surface receptor for *Plasmodium falciparum*-infected erythrocytes. *J. Exp.Med.* 182,

10     15-20). Taken together this may indicate that the parasite has adapted a more general
strategy for interacting with the host by using the negatively charged proteoglycans as
receptors exposed on the exterior of every cell surface.

According to the present invention, it has also been found that the rosette disruption

15     capacity is not only determined by the molecular weight of SGs. Hyaluronic acid, by
far the largest of all tested molecules, exhibited no rosette disrupting activity while
smaller molecules did. Also, an association between average negative charge and
rosette reverting capacity was not always observed, e.g. hyaluronic acid which has a
higher average negative charge density than fucoidan did not exhibit a rosette-

20     disrupting activity while fucoidan did. Comparison of heparin and fucoidan, on the
other hand, suggests that the effect of correct positioning within a three dimensional
structure could be much more essential than the average amount of negative charge
which is clearly lower in fucoidan (Patankar *et al.* 1993). An association to negative
charge could be the explanation for differences between heparin and HS, although

25     more specific sequence determinants can not be excluded.

When assaying the heparin/HS-sensitive FCR3S1 strain it is apparent that modified
heparin preparations with reduced overall degree of sulfation, and thus negative
charge, loose competition capacity though not in a strictly charge dependent manner.

30     This finding supports the conclusion that other determinants must be of importance for

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the binding to infected erythrocytes. *N*-desulfated heparin preparations were at least one hundred times weaker rosette reverters comparted to heparin. In contrast to the effect of *N*-desulfation, *O*-desulfation seemed to be connected with minor reduction of competition efficiency, indicating the importance of the type of sulfate, *i.e. N*-sulfation.

5   An analogous conclusion may be drawn by comparison of the more efficient rosetting reverter heparin as compared to HS. One of the basic differences between heparin and HS is the degree and distribution of the *N*-sulfate groups, which in turn leads to a predominant localization of *O*-sulfation to *N*-sulfated blocks (Salmivirta *et al.* 1996). *N*-sulfation reaches 40 - 60 % of all the disaccharides in an average HS chain

10  (Maccarana *et al.* 1996) while the value for heparin is over 90 %. Indeed, the difference in competition offect of heparin and HS in rosetting could be explained by the more abundant presence of *N*-sulfation in heparin.

To further test the importance of sulfation position over anionic feature of a

15  carbohydrate, heparin/HS derived mono- and disaccharides were assayed for rosette disrupting activity. Twenty-nine wild isolates were tested for sensitivity to heparin and heparin disaccharide I-S ($\Delta$HexA(2-OSO$_3$)-GlcNSO$_3$-(6-OSO$_3$)), and the sensitivity pattern obtained was very similar for the two compounds at equipotent concentrations, whereas it could differ for other GAGs and polysaccharides. Our results talk therefore

20  in favour of an identical site of action for heparin and the heparin disaccharide I-S suggesting that results with mono- and disaccharides reflect at least part of the heparin/HS binding site(s) on infected erythrocytes.

Molecular net negative charge can not by itself explain the anti-rosetting activity: some

25  highly negatively charged monosaccharides exhibited no rosette disrupting effect, while some monosaccharides with one single negative charge disrupted rosettes. Also, the fact that higher degree of sulfation did not increase rosette reverting potency, corroborates that it is the position of the sulfate group(s) rather than the number that is the determinant. In case of the disaccharides, 6-*O*-sulfation seems to be less important,

30  while the 2-*O*-sulfate has some positive effect in addition to the *N*-sulfate group. This

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could be explained by rotational freedom of the glycosidic linkage which results in the possible exposure of both sulfate groups on the same side of the sugar plane, while the 6-$O$-sulfate would protrude on the opposite side. Also, $N$-sulfated glucosamine (present in heparin and HS) abolishes rosetting, whereas its stereoisomer $N$-sulfated

5    galactosamine (not present in any GAG) does not, all emphasizing that the $N$-sulfated glucosamine is part of the binding sequence.

To analyze whether competition by heparin and heparin derived oligosaccharides reflects the existence of a heparin/HS-like ligand on the erythrocytes, whole cultures

10   and uninfected erythrocytes were treated by different glycan degrading procedures. For the heparin/HS sensitive strain FCR3S1 a clear reduction of rosette formation was provoked by either unspecific glycan oxidation through periodate or a selective digestion of heparin/HS chains by heparinase (Fig. 6). Rosetting was not hampered by either chondroitinase, neuraminidase or phospholipase C treatment of cultures,

15   supporting the view that the ligand is of heparin/HS nature. Proteolytic treatment of the cultures, on the other hand, indicated that protein components are involved in the rosetting mechanism as well, either as lectin-lika ligands (i.e. on the infected erythrocyte) or as carrier of the glycan epitopes (i.e. on the uninfected erythrocyte). Rosetting by strain TM 284 seemed to be more sensitive to competition by chondroitin

20   sulfate and dermatan sulfate, although higher amounts of heparin/HS were also effective. This observation was confirmed by enzymatic treatment of the erythrocytes as chondroitinase had a profound effect on the rosetting.

The separate treatment of uninfected erythocytes was performed in order to establish

25   whether the infected erythrocyte or the rosetting uninfected erythrocytes contain specific features involved in rosetting. The higher sensitivity of the uninfected erythrocytes for glycan destruction would indicate that glycan structures constitute parts of the rosetting receptor(s). The opposite argumentation would hold true for the proteolytically destroyed component which seems to sit on the side of the infected

30   erythrocytes, although also a part of the glycans could be removed by proteolytic

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cleavage of their core proteins. However, the infected erythocytes seem to be much more sensitive for the proteolytic degradation as compared to glycan oxidation.

We have previously suggested that rosetting may be mediated by lectin-like interactions, with non-obligate involvement of different ABO-blood group antigens (Carlson *et al.* 1992). CD36 has also been attributed a receptor function (Handunnetti *et al.* 1992) and recently, complementreceptor 1 was reported to play a role in the fomration of rosettes (Rowe *et al.* 1997). The fact that heparin sensitivity decreases when the same parasites are cultivated in erythrocytes from their preferred ABO-blood group (Carlson *et al.* 1992) could indicate simultaneous involvement of more than one rosetting receptor and that heparin is not interacting with these receptors. A role for serum proteins in rosetting has also been shown (Scholander *et al.* 1996). Although an interaction of GAGs with serum factors can not be excluded, this effect should be of minor importance since strains with different serum dependency were equally affected (not shown). Furthermore, a recombinant fusion-protein covering the C-terminally located Duffy-like binding domain (DBL-1) of PfEMP1, adhered directly to erythrocytes and bound to a heparin-matrix, in the absence of serum factors (Chen *et al.* 1998).

As a conclusion, the present invention provides the identification, by single-cell RT-PCR and cDNA cloning, of a new adhesive ligand *Plasmodium falciparum* erythrocyte membrane protein-1-variant , PfEMP1. Rosettes of the novel PfEMP1-variant contains clusters of glycosaminoglycan-binding motifs. A recombinant fusion protein (DBL-1-GST) has been found to adhere directly to normal erythrocytes, disrupt naturally formed rosettes, block rosette-reformation and bind to a heparin-sepharose matrix. The adhesive interactions could be inhibited with heparan suifate or enzymes that remove heparan sulfate from the cell surface while other enzymes or similar glycosaminoglycans of an alike negative charge did not affect the binding. Further, fucoidan, a sulfated glycan, has been shown to be particularly effective in disrupting rosettes. The novel PfEMP1-variant is suggested to be the rosetting ligand and

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carbohydrates, heparan sulfate, or a heparan sulfate-like molecule, the receptor both for PfEMP1 binding and naturally formed erythrocyte rosettes.